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<p>(21) International Application Number: PCT/US90/05106</p> <p>(22) International Filing Date: 13 September 1990 (13.09.90)</p> <p>(30) Priority data: 413,274 27 September 1989 (27.09.89) US 577,650 4 September 1990 (04.09.90) US</p> <p>(71) Applicant: ATHENA NEUROSCIENCES, INC. (US/US); 800F Gateway Boulevard, South San Francisco, CA 94080 (US).</p> <p>(72) Inventors: RUBIN, Lee, L. ; 122 Funston Avenue, San Francisco, CA 94118 (US). PORTER, Seth ; 913 Fassler Avenue, Pacifica, CA 94044 (US). HORNER, Heidi, C. ; 150 Duane Street, #9, Redwood City, CA 94062 (US). YEDNOCK, Theodore, A. ; 46 Crest Road, Fairfax, CA 94030 (US).</p>		<p>(74) Agents: BLECHER, Melvin et al.; 611 West Sixth Street, 34th Floor, Los Angeles, CA 90017 (US).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: BLOOD-BRAIN BARRIER MODEL</p> <p>(57) Abstract</p> <p>An <i>in vitro</i> model of a blood-brain barrier comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells in contact with agents that elevate effective cyclic AMP concentrations in endothelial cells, with or without astrocyte-derived or endothelial cell-derived conditioned medium or the equivalent so that high electrical resistance tight junctions are formed between endothelial cells, and endothelial cells exhibit peripheral phalloidin staining and E-cadherin. Also disclosed is the use of agents that reduce effective cyclic AMP concentrations or interfere with the functioning of cyclic AMP or increase effective cyclic GMP concentrations to open up blood-brain barriers <i>in vitro</i> and <i>in vivo</i>, so that drugs normally excluded by such barriers may substantially penetrate such barriers. Also disclosed are uses of the model to screen for reagents with clinical utility in disorders involving brain endothelial cells.</p>		

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## BLOOD-BRAIN BARRIER MODEL

This is a continuation-in-part of United States Serial No. 097/413,274, filed September 27, 1989.

BACKGROUND OF THE INVENTIONField of the Invention

5       The present invention relates generally to in vitro models of endothelial cells. More particularly, the invention relates to an in vitro model that simulates the characteristics of microvascular endothelial cells of the brain that constitute the  
10   blood-brain barrier.

Description of Related Art

The vertebrate brain has a unique capillary system unlike that of any other organ of the body. This unique capillary system has morphological and  
15   biochemical characteristics that make up the "blood-brain barrier" (BBB). The BBB acts to separate the brain interstitial space from the blood. This barrier prevents molecules in the blood that are neither lipophilic or transported by specific carrier proteins  
20   from entering the brain (Betz, A.L. et al., Ann. Rev. Physiol., 48:241 (1986); Pardridge, W.M., Ann. Rev. Pharmacol. Toxicol., 28:25 (1988)).

The characteristics of the brain capillaries that make up the BBB include: (a) high-resistance tight  
25   junctions between endothelial cells of the brain that block transport of molecules between cells; and (b) limited amount of transport across cells, as compared to that occurring in peripheral capillaries.

0       The tight junctions of the BBB prevent passive  
diffusion of molecules and ions around the endothelial  
cells. Thus, most hydrophilic drugs and peptides that  
gain ready access to other tissues of the body are  
barred from entry into the brain, or their rates of  
5 entry are low. Thus, at the BBB, the only substances  
that can readily pass from the luminal core of the  
capillary to the abluminal tissue that surround the  
capillary are those molecules for which selective  
transport systems exist in the endothelial cells, as  
10 well as compounds that are lipid soluble. Such  
compounds, because of their inherent lipophilicity, are  
able to intercalate into the plasma membrane of  
endothelial cells and move to the abluminal side.  
These unique properties of the BBB have provided a  
15 major hindrance to the development of therapeutic  
agents directed toward diseases of the central nervous  
system (CNS), e.g., Alzheimer's disease and Parkinson's  
disease.

      There are two general situations in which the  
20 ability to test for CNS entry of therapeutic agents is  
important. First, the increasing prevalence of CNS  
disorders and the introduction of new molecular  
biological and biochemical techniques to treat such  
disorders will lead to the development of new drugs  
25 that will be centrally active. These drugs must be  
tested for their ability to reach the brain, i.e.,  
penetrate the BBB. Second, many drugs used to treat  
peripheral disorders have undesirable CNS side effects.  
As replacements for these drugs are developed, they  
30 will have to be screened for CNS penetration as well.  
Of course, the objective in that case is to develop  
peripherally-acting drugs that do not enter the brain.



0        Screening batteries of compounds for passage into  
the brain by conventional techniques is impractical.  
Generally, compounds are introduced into the carotid  
artery, and their concentration in the brain is then  
determined. This means that for each individual  
5    compound many animals must be injected and processed.  
While animal testing in vivo is important, it is not  
the optimal screening system when many compounds have  
to be examined.

Thus, it would be highly desirable to have an in  
10 vitro model of the BBB so as to be able efficiently and  
inexpensively to screen numerous drugs in a relatively  
short amount of time. The test system should closely  
simulate the morphological and physiological  
characteristics of the in vivo BBB in having tight  
15 junctions between cells and similar permeability  
characteristics, and should be composed of defined cell  
types.

Another desirable characteristic of an in vitro  
model is that it should provide a system for testing  
20 manipulations of the endothelial cells of a nature as  
to increase or decrease the passage of drugs from the  
blood side to the brain side of these cells.

Previous attempts to construct an in vitro model  
of the BBB have not met the criteria outlined above.  
25 Intact brain microvessels (Kumagai, A.K., J. Biol.  
Chem., 262:15214 (1987)) are likely to contain not only  
endothelial cells and astrocytes, but mast cells as  
well. Further, the limited volume and access to the  
lumen of microvessels precludes their use for vectorial  
30 transport studies, and therefore makes them suboptimal  
as a workable model for the BBB.

Several laboratories claim to have created a BBB  
in vitro model using brain capillary endothelial cells

0 in the presence of standard growth media (Audus, K.L.,  
et al., Ann. N.Y. Acad. Sci., 507:9 (1987); Van Bree,  
J.B.B.H., et al., Pharm. Res., 5:369 (1988); Hart,  
M.N., et al., J. Neuropath. Exp. Neurol., 46:141  
(1987)). Cloned bovine brain capillary endothelial  
5 cells, grown on a permeable support of glutaraldehyde-  
treated collagen gel, have been reported to exhibit  
high transendothelial cell resistance (Rutten, M.J.  
et al., Brain Res., 425:301 (1987)). However, these  
studies have demonstrated only one or a few of the  
10 inherent morphological, biochemical and functional  
characteristics of brain capillaries, and the data  
derived from such systems are often conflicting, in  
part because in most studies the systems employed  
incompletely characterized populations of primary cell  
15 cultures or cell lines, and in part because the brain  
capillary endothelial cells were not grown in the  
proper milieu.

It is known that brain astrocytes influence the  
properties of brain capillary endothelial cells.  
20 Janzer et al. (Janzer, R.C., Nature, 325:253 (1987))  
disclosed that neonatal rat brain type 1 astrocytes,  
cultured on filters and transplanted into the eyes of  
syngeneic animals or chick embryo chorioallantoic  
membranes, became vascularized by the endogenous  
25 endothelial cells, and caused the endothelial cells to  
exclude the dye, Evans blue.

Exclusion of Evans blue dye or other cationic dyes  
that bind to albumin is one property of endothelial  
cells in the brain. These results might be used to  
30 predict that astrocytes can cause endothelial cells to  
exhibit a generally low rate of macromolecular  
transport. They do not necessarily indicate, however,  
that the endothelial cells have been induced to form

0 the high resistance tight junctions which are also characteristic of those cells in vivo.

Other in vitro studies have examined the effects of brain astrocytes on ultra-structural properties of endothelial cells. Brain astrocytes enhanced the  
5 frequency, length and complexity of tight junctions formed between cultured, brain-derived endothelial cells (Tao-Cheng, J.-H. et al., J. Neurosci., 7:3293 (1987)). Also, fourth passage rat brain capillary endothelial cell cultures, grown in rat brain  
10 astrocyte-conditioned medium on endothelial cell matrix-coated substrate, exhibited tight junction biogenesis (Arthur, F.E. et al., Dev. Brain Research, 36:155-9 (1987)). Both studies relied solely upon  
15 ultrastructural examination of individual groups of treated cells, but neglected to look at resistance of tight junctions.

Thus, an important need still exists for an in vitro model of a BBB that meets all of the criteria necessary for a model to simulate the in vivo  
20 situation: 1) a monolayer of endothelial cells essentially all of which are connected by tight junctions; 2) a diffusion barrier for components that do not ordinarily cross the BBB; and 3) a high transendothelial cell electrical resistance barrier  
25 indicating the presence of tight junctions that prevent passive diffusion of ions.

#### SUMMARY OF THE INVENTION

In accordance with the present invention, an in vitro model of the vertebrate BBB is disclosed that  
30 simulates important morphological and permeability characteristics of the brain BBB, that permits the

0 efficient and inexpensive screening of CNS drugs, and  
that allows testing of manipulations of the BBB.

The present invention is based on the effects of  
the brain microenvironment on the special properties of  
brain capillary endothelial cells. More specifically,  
5 the present invention is based in part upon a  
reconstruction of interactions between brain astrocytes  
and brain capillary endothelial cells in in vitro  
monolayer systems.

This invention is also based on the discovery that  
10 treatments that raise effective cyclic AMP  
concentrations in cultured brain endothelial cells,  
particularly in conjunction with the presence of  
components of endothelial cell and brain astrocyte-  
derived conditioned media or the equivalent, markedly  
15 increase the production of tight junctions that exhibit  
properties of the BBB in vivo such as high electrical  
resistance, peripheral staining of cells by phalloidin,  
and a diffusion barrier for substances known ordinarily  
not to cross the BBB.

20 This invention is also based upon the discovery  
that tight junctions between brain microvascular  
endothelial cells may be disrupted and the blood-brain  
barrier made more permeable by agents that decrease the  
effective intracellular concentration of cyclic AMP,  
25 interfere with the physiological actions of cyclic AMP,  
increase the effective intracellular concentration of  
cyclic GMP, or promote the physiological actions of  
cyclic GMP, and that such manipulations allow for the  
facile delivery of drugs across the blood-brain  
30 barrier.

It is thus an object of this invention to disclose  
an in vitro model of the BBB comprising a porous solid  
support separating monolayer cocultures of

0 microvascular endothelial cells and brain astrocytes  
juxtaposed in a device that permits physiological  
interaction between the cell types.

It is another object of this invention to disclose  
an in vitro model of the BBB comprising a monolayer of  
5 microvascular endothelial cells disposed on a filter in  
contact with conditioned growth media derived from  
endothelial cells or astrocytes.

It is yet another object of this invention to  
provide criteria for selecting endothelial cells and  
10 astrocytes particularly suitable for the in vitro  
model.

It is a further object of this invention to  
provide criteria for selecting substrata for the  
culture of monolayers of cells in the in vitro model of  
15 the invention.

It is still another object of this invention to  
provide means for elevating the effective intracellular  
cyclic AMP concentration in microvascular endothelial  
cells of the in vitro model.

20 It is yet another object of this invention to  
provide testing criteria for the genesis of tight  
junctions in the in vitro model of the invention.

It is yet another object of this invention to  
provide an in vitro model of a BBB which uses  
25 endothelial cells from blood vessels other than brain  
capillaries.

It is still another object of this invention to  
provide compositions and methods involving  
manipulations of cyclic AMP and cyclic GMP levels or  
30 physiological effectiveness in order to open up blood-  
brain barriers in vivo and in vitro and thereby to  
permit drug delivery across such barriers.

0        These and other objects of this invention will  
become clear by reference to the following disclosure  
and appended claims.

#### DESCRIPTION OF THE FIGURES

5        Fig. 1 provides transmonolayer electrical  
resistance data for the BBB model of the invention  
using bovine brain capillary endothelial cell cultures.

Fig. 2 provides albumin flux data for monolayer  
cultures of bovine retinal endothelial cells and MDCK  
epithelial cells.

10        Fig. 3 shows flux data for sucrose and  
chlorambucil across tight junction brain endothelial  
cells.

15        Fig. 4 shows the effect of agents that lower  
cyclic AMP concentrations on morphine analgesia in  
animals.

Fig. 5 shows the effects of elevating endothelial  
cell levels of cyclic GMP on cyclic AMP-induced tight  
junctions.

20        Fig. 6 shows the effects of elevating in vivo  
levels of cyclic GMP on morphine analgesia.

Fig. 7 shows the effects of different classes of  
cyclic AMP phosphodiesterase inhibitors on tight  
junctions of brain endothelial cells in the BBB in  
vitro model.

0        Fig. 8 Panels A and B, are photomicrographs of  
sections from a brain sections in which MS-type  
inflammation was induced via intracranial injection of  
human tumor cells. Human and mouse lymphocytes were  
allowed to contact the sections, and, as seen in Panel  
5    A, bind selectively to exposed brain endothelium. In  
Panel B, the lymphocytes were treated with an antibody  
that inhibits the human VLA-4 receptor (anti-human  $\beta$ -1  
integrin) and as can be seen, the human lymphocyte  
(large cell) binding is substantially inhibited.

10       Fig. 9 is a photograph showing lymphocyte binding  
and inhibition to binding in brain endothelial cells in  
culture. Panel A shows the low level binding of  
lymphocytes to the BBB model endothelium. In Panel  
B, the endothelium has been treated with an inflammatory  
15    reagent, and lymphocyte binding is increased  
substantially. In Panel C, the lymphocytes were  
pretreated with anti-human  $\beta$ -1 integrin monoclonal  
antibody, and their binding to the stimulated  
endothelium is substantially inhibited.

20       Fig. 10 is a graph showing the relative degree of  
lymphocyte binding to blood vessels in sections of  
inflamed brain tissue, and, as a basis for comparison,  
normal lymph node tissue. The "no additions" column  
shows brain tissue (scored) and lymph node endothelium  
25    (solid) to which untreated lymphocytes have been added.  
The degree of binding is represented as 100%. In the  
next two columns, lymphocytes have been pretreated with  
anti-VLA-4 reagents. The middle bars show lymphocytes  
pretreated with anti- $\beta$ -1 monoclonal antibody, the  
30    right-hand bars show lymphocytes pretreated with anti-  
 $\alpha$ -4 monoclonal antibody. In both cases, lymphocyte  
binding in brain tissue is almost completely inhibited,  
as compared to the control. But, lymphocyte binding to

0 lymph node endothelium, in both cases, is not significantly inhibited.

Fig. 11 is a graph showing the relative degree of Jurkat T-cell lymphocyte binding to brain endothelial cells in the BBB system. As can easily be seen, the anti- $\beta$ -1 antibody effectively inhibited the binding of leukocytes to TNF- $\alpha$  activated brain endothelial cells. Anti- $\beta$ -2, as a control, on the other hand, approaches the untreated control. Plainly, the  $\beta$ -1 subunit provides an effective target for preventing VLA-4/VCAM-1 interaction in the brain.

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#### DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises an in vitro model of mixed or cloned endothelial cells expressing high electrical resistance tight junctions and other properties of the BBB in vivo.

15

One embodiment of the invention comprises a chamber separated into at least two compartments by a porous solid support, on one surface of which support is disposed an essentially confluent monolayer of brain microvascular mixed or cloned endothelial cells growing on a particular substratum, the second compartment of the chamber housing an essentially confluent monolayer of brain astrocytes disposed either on a second surface of the chamber or on the underside of the porous solid support, the monolayers of both cell types being in sufficiently close juxtaposition so that products of each cell type can readily reach the cells of the other cell type. By "porous" we mean containing interstices through which water and the solutes contained therein, but not cells, can pass. In an alternative embodiment, the growth medium in contact with the endothelial cells contains, in part, an astrocyte or endothelial cell-

20  
25  
30



0 derived conditioned medium or equivalent. By  
"conditioned medium" we mean a tissue culture growth  
medium into which cultured cells have secreted  
materials of cellular origin. By "equivalent" we mean  
5 cell or tissue extracts containing materials of  
cellular origin that may in other circumstances be  
secreted extracellularly. Examples of the preparation  
of conditioned mediums and equivalents are provided  
below. In these embodiments of the model,  
transcellular electrical resistance can be measured  
10 directly, as described below. Details as to the  
construction of these models will be provided below.

In another embodiment of the invention, mixed or  
cloned microvascular endothelial cells may also be  
grown on coated microcarrier beads, e.g., Cytodex-3  
15 microcarriers (Pharmacia, Uppsala, Sweden), according  
to Ryan et al. (Ryan, J., et al., Tissue Cell, 12:619  
(1980)). Although in this model transcellular  
resistance cannot be measured directly, macromolecular  
transcellular transport, e.g., of labeled albumin,  
20 cationized albumin, or glycosylated albumin, and of  
dyes such as Trypan blue or Evans blue, can be  
determined (Kempski, O., et al., Acta Neuropathol.,  
74:329 (1987); Bioadjieva, S., et al., Lab Invest.,  
50:239 (1984); Smith, R.K., et al., Pharm. Res., 5:466  
25 (1988)). The influence of astrocytes on endothelial  
cells grown on microcarriers can be determined by first  
growing brain astrocytes, such as neonatal rat type 1  
astrocytes, on the beads, then removing the astrocytes,  
leaving their extracellular matrix behind. This can be  
30 accomplished either by lysing the astrocytes in 5 mM  
Tris buffer, pH 7.4, containing 1% Triton X-100 for 15  
minutes or by incubating the astrocytes in phosphate-  
buffered saline (PBS) containing 10 mM EDTA for 30

0 minutes, both solutions containing protease inhibitors,  
e.g., aprotinin and phenylmethanesulfonyl fluoride.  
Coated beads can be washed 3 times in PBS, then treated  
with 25 mM  $\text{NH}_4\text{OH}$ . After the beads are washed again  
with PBS, they can be coated with an essentially  
5 confluent layer of cultured endothelial cells. Once  
the endothelial cells reach confluence, they can be  
maintained in a growth medium containing cultured  
astrocytes, or in astrocyte-derived or endothelial  
cell-derived conditioned medium.

10 In another embodiment, mixed or cloned  
microvascular endothelial cells can be grown on porous  
tube-like structures, such as those used in hollow-  
fiber cell growth devices (Amicon Corp., Danvers, MA).  
Again, a surface of hollow fibers can be coated with  
15 astrocytes, from which astrocyte extracellular matrix  
(ECM) can be prepared as described above. Endothelial  
cells can then be grown on the astrocyte ECM, and the  
cells exposed to astrocyte or endothelial cell-derived  
conditioned medium. In this embodiment, transcellular  
20 electrical resistance can be measured by passing  
current between electrodes inside and outside the  
hollow fiber. Macromolecular flux can be measured by  
adding labeled macromolecules outside the fiber, and  
following their transport across the endothelial cells  
25 into the fiber.

#### Origin of Astrocytes

Purified populations of neonatal rodent brain  
type 1 astrocytes were prepared according to the  
procedures of Lillien et al. (Lillien, L.E., et al.,  
30 Neuron, 1:485 (1988)). In brief, cerebral cortices  
were removed from neonatal rats, white matter was  
discarded, and the gray matter mechanically and

0 enzymatically (trypsinization) dissociated. Cells were  
plated in poly-lysine-coated flasks in Dulbecco's  
Modified Eagle's Medium (DMEM) plus 10% fetal calf  
serum (FCS). After 5 days, loosely attached cells were  
dislodged by shaking, attached cells were passaged into  
5 new flasks, and treated with cytosine arabinoside (an  
anti-mitotic drug) to remove actively proliferating  
contaminating cells. Finally, astrocytes were  
maintained in a chemically defined medium and fed twice  
weekly. Cell type was determined by reactivity with  
10 particular sets of antibodies. For example, type 1  
astrocytes are fluorescently labeled by an antibody  
against glial fibrillary acidic protein, but not with  
the monoclonal antibody A2B5 (which labels type 2  
astrocytes) or with an anti-galactocerebroside antibody  
15 (which labels oligodendrocytes) (Raff, M.C., et al.,  
J. Neurosci., 3:1289 (1983)).

#### Origin of Capillary Endothelial Cells

Endothelial cells are prepared from a variety of  
animal and human sources. For example, mixed  
20 populations of endothelial cells may be prepared from  
purified capillaries derived from rodent and bovine  
brain, bovine retina, bovine adrenal, bovine aorta, and  
human omentum or from human umbilical vein. Bovine  
sources are particularly suitable because of the large  
25 amounts of tissue available, the ready availability of  
fresh tissues, and the similarity of the permeability  
of bovine capillary cells to that of their human  
counterparts.

Bovine brain microvascular cells were isolated  
30 according to Audus et al., Pharm. Res., 3:51 (1986)).  
Briefly, a slurry of brain grey matter in Liebovitz'  
L-15 medium was homogenized, and the microvascular

0 cell-containing particular fraction was separated on  
a Dextran cushion. Capillaries were resuspended and  
homogenized, then passed through a series of nylon  
filters. Capillaries were digested further with  
collagenase plus trypsin to provide a population of  
5 single mixed endothelial cells. These cells were  
plated on a collagen or fibronectin treated substratum  
in 10% plasma-derived horse serum (PDHS) in Dulbecco's  
modified Eagle's Medium (DMEM). Rat brain  
microvascular endothelial cells were prepared similarly  
10 according to Bowman, et al. (Bowman, P.D., et al., In  
Vitro, 17:353 (1981)). Briefly, brain grey matter is  
minced and digested with collagenase and dispase. The  
particulate matter is separated over a 25% bovine serum  
albumin (BSA) cushion, and the pellet further digested  
15 with collagenase and DNase. Finally, endothelial cells  
are isolated on a Percoll gradient, and washed cells  
are plated on a collagen-treated substratum in DMEM +  
20% plasma-derived horse serum (PDHS) + 150 µg/ml  
endothelial cell growth supplement (ECGS, available  
20 from Sigma Chemical Co., St. Louis, MO); (McGuire,  
P.G., et al., Lab. Invest., 57:94 (1987)).

To prepare mixed bovine aortic endothelial cells,  
aortas were trimmed of adventitia and connective  
tissue, cut open to expose the intimal layer, and the  
25 internal aspect contacted with 0.1% collagenase in RPMI  
1640. After incubation for 20 minutes at 37°C, the  
loosened cells were scraped into DMEM + 10% fetal calf  
serum (FCS) and plated into tissue culture flasks. For  
rodent aortic endothelial cells, the exposed intima  
30 were placed on a collagen-treated surface in a minimal  
quantity of growth medium (DMEM + 20% FCS + 150 µg/ml  
ECGS); endothelial cells will grow out from the explant  
and proliferate in this growth medium.

0       Mixed populations of human endothelial cells may  
also be isolated from fresh umbilical veins. After  
cannulating the vein and flushing it with RPMI 1640  
medium, the intimal layer is exposed to 1 mg/ml  
collagenase in RPMI 1640. After 15 minutes at about  
5   37°C, the detached cells are washed out of the vein,  
pelleted by centrifugation, the cell pellet suspended  
in DMEM + 20% FCS, and the cells plated on a collagen-  
treated substratum (Gimbrone, M.A., et al., J. Cell  
Biol., 60:623 (1974)). These cells are also available  
10 commercially (Clonetics, San Diego, CA).

Cells are identified as endothelial by  
immunofluorescence assay with anti-von Willebrand  
protein (rabbit serum from Bering Diagnostics, La  
Jolla, CA) and uptake of di-I-labeled acetylated LDL  
15 (Molecular Probes, Junction City, OR). Endothelial  
cells are typically passaged once a week and maintained  
in DMEM + 10% or 20% FCS or 10% PDHS.

Endothelial cell cultures can be cloned, if  
desired, using the cloning ring technique. Cells are  
20 plated in at low density (1000 cells per 10 cm plate)  
in 10% FCS. Plastic cloning rings, dipped in silicone  
grease, are paced on cells so as to encircle and  
isolate single or paired cells on an inverted  
microscope. Once the clone expands, the cells are  
25 detached by trypsinization within the ring and  
transferred to a well of a multi-well culture disk.  
Multiple clones of microvascular endothelial cells from  
bovine brain, bovine aorta, rat aorta, and rat brain  
can be isolated by this technique.

### 30   Astrocyte-Derived Conditioned Medium

Neonatal rat brain type 1 astrocytes were grown to  
confluency in poly-D-lysine-coated 75 cm<sup>2</sup> flasks.

- 0 Fresh medium was added to the cells, and removed after 2-4 days. The medium was filtered through a 0.2  $\mu$  Millipore filter, and stored frozen at -80°C in small aliquots.

#### Endothelial Cell-Derived Conditioned Medium

- 5 Bovine aortic or retinal endothelial cells were grown to confluency in 75 cm<sup>2</sup> flasks. Fresh medium was added to the cells, and conditioned medium collected and stored as above.

#### Astrocyte Extract

- 10 Type 1 astrocytes from neonatal rat brain were grown as above. Cells were scraped from the dish in 3 ml of ice-cold DMEM, and homogenized in a Dounce homogenizer at ice-bath temperatures. After centrifuging the homogenate at 40,000 rpm for 30  
15 minutes in a Beckman Instruments SW40 rotor, the supernatant fluid was filtered through a 0.2  $\mu$  Millipore filter, and small aliquots stored frozen at -80°C.

#### Brain Extract

- 20 Neonatal rat brain cortex was removed and homogenized in DMEM (3 ml per gm. tissue, wet wt.) in a Dounce homogenizer. The homogenate was centrifuged and processed as for the astrocyte extract above.

#### Elevation of Cyclic AMP Concentrations

- 25 Cultures of endothelial cells were treated with one or more agents known to increase cyclic AMP concentrations. These include, but are not limited to:  
1) from about 10 to about 100  $\mu$ M of a  $\beta$ -adrenergic agent, such as isoproterenol, that binds to specific

0  $\beta$ -adrenergic receptors on cell surfaces and stimulates  
G-protein-mediated activation of adenylate cyclase; 2)  
serotonergic compounds such as 5-hydroxytryptamine; 3)  
forskolin, (Sigma Chem. Co., St. Louis, MO) an agent  
that directly activates adenylate cyclase; 4)  
5 parathyroid hormone; and 5) calcitonin gene related  
peptide. Adding an inhibitor of cyclic AMP  
phosphodiesterase, the enzyme that degrades cyclic AMP  
to adenylic acid, will accentuate the cyclic AMP  
elevating effects of the aforementioned modalities;  
10 examples of such inhibitors are 4-(3-butoxy-4-  
methoxybenzyl)-2-imidazolidinone (Hoffman-LaRoche,  
Nutley, N.J.), theophylline and methylisobutylxanthine  
(Sigma Chem. Co.), Rolipram (Berlex, Inc.) and  
RO-20-1724 (BioMol, Inc., Phymouth Meeting, PA). In  
15 addition, certain derivatives of cyclic AMP can be used  
to elevate the effective cyclic AMP concentration in  
such cells; such derivatives include 8-bromo cyclic AMP  
(Sigma Chem. Co) and 8-(4-chlorophenylthio)cyclic AMP  
(Boehringer-Mannheim Corp., Indianapolis, IN). By  
20 "effective cyclic AMP" we mean endogenous cyclic AMP or  
cyclic AMP derivatives to which endothelial cells are  
permeable and which act physiologically as does  
endogenous cyclic AMP within such cells. By "effective  
cyclic GMP" we mean endogenous cyclic GMP or cyclic GMP  
25 derivatives to which endothelial cells are permeable  
and which act as does endogenous cyclic GMP within such  
cells. By "physiological action" of cyclic AMP or  
cyclic GMP or derivatives thereof we mean those  
immediate biochemical reactions of these cyclic  
30 nucleotides that lead ultimately to the physiological  
actions ascribed to them. For example, cyclic AMP  
activates certain protein kinases that catalyze the  
phosphorylation of hydroxyamino acid residues such as

0 serine, threonine and tyrosine in particular proteins,  
such phosphorylation activating these proteins.  
Effects of cyclic AMP are reversed by phosphoprotein  
phosphatases that catalyze the de-phosphorylation of  
the aforementioned hydroxyamino acid residue-containing  
5 proteins.

When brain capillary endothelial cells were grown  
on a porous solid support with a growth medium  
containing PDHS plus one or more of the aforementioned  
agents that elevated the actual or effective  
10 intracellular cyclic AMP concentration, transmonolayer  
electrical resistance increased about 7-fold, from  
about 50 to about 350 ohm-cm<sup>2</sup> (Fig. 1). However, when  
in this system, bovine aortic endothelial cell-derived  
conditioned medium (BAEC-CM) was also present, the  
15 transmonolayer electrical resistance increased about  
10-fold (Example 5). Growing endothelial cell  
monolayers on astrocyte extracellular matrix  
potentiated the effects of cyclic AMP and BAEC-CM,  
producing as much as a 26-fold increase in resistance  
20 (Example 5). Thus, the BBB model of this invention is  
capable of providing transmonolayer electrical  
resistances of at least 200 ohm-cm<sup>2</sup>, preferably greater  
than about 300 ohm-cm<sup>2</sup>, more preferably greater than  
about 1000 ohm-cm<sup>2</sup> up to about 1500-2000 ohm-cm<sup>2</sup>.

25 In addition, it has been discovered that an  
elevation of actual or effective cyclic AMP  
concentrations, with consequent formation of tight  
junctions between mixed endothelial cells as determined  
by electrical resistance measurements, was also  
30 associated with substantial peripheral staining by  
phalloidin, a toxin produced by Amanita phalloides that  
is known to bind to filamentous actin and prevent their  
depolymerization (Stryer, L., "Biochemistry", 3d., W.H.



0 Freeman, N.Y. 1988, p. 940). The belt-like pattern of  
phalloidin staining in these treated endothelial cells  
is similar to that seen in epithelial cells exhibiting  
high resistance tight junctions. (Gumbiner, B., J.  
5 Cell Biol., 107:1575 (1985).) In addition, when  
endothelial cells were grown with endothelial cell-  
derived or astrocyte-derived conditioned medium plus  
cyclic AMP enhancing agents such that peripheral  
phalloidin staining of cells was substantially present,  
10 transmonolayer electrical resistance was increased over  
that obtained in the absence of conditioned medium.

It has also been discovered that the formation of  
tight junctions between endothelial cells in the blood-  
brain barrier model of the invention is substantially  
enhanced when endothelial cells are grown from the time  
15 of their isolation in the presence of astrocyte-derived  
conditioned medium (ADCM). Thus, when endothelial  
cells are passaged onto filters, e.g., Costar filters,  
they are preferably grown in a medium containing 50%  
ADCM made in MEM with 10% fetal calf serum and 50% N2  
20 (a chemically-defined medium). After 2-3 days of  
growth on the filters, they may be treated with a  
cyclic AMP analogue and a cyclic AMP phosphodiesterase  
inhibitor (e.g., Rolipram or RO 20-1724).

In addition, it has been discovered that, when  
25 endothelial cells are cultured in concentrations of  
fetal calf serum substantially lower (e.g., 0.5% to 5%)  
than the customary 10% used for culturing such cells  
for other purposes, increased cellular resistivity in  
the blood-brain model of the invention may be attained.

0    Decreased Cyclic AMP Concentrations  
     or Physiological Activity

     As noted above, increases in cyclic AMP in tissues by whatever means (e.g., addition of cyclic AMP analogues, addition of compounds that stimulate  
5    endogenous adenylate cyclase activity, or addition of compounds that inhibit the activity of cyclic AMP phosphodiesterase thereby inhibiting cyclic AMP degradation) result in enhanced tight junction formation between brain endothelial cells.

10       It has also been discovered that removal of cyclic AMP analogues or other agents that elevate cyclic AMP levels from endothelial cell cultures in the blood-brain model of the invention produces a rapid decrease in resistance, signalling increased permeability of  
15    tight junctions.

     These discoveries have led to other approaches to regulating tight junctions between brain endothelial cells: (a) compounds that inhibit formation of endogenous cyclic AMP by adenylate cyclase; (b)  
20    competitive inhibitors of cyclic AMP; (c) inhibitors of protein kinases, the enzymes that are activated by cyclic AMP; and (d) stimulators of protein phosphatases, the enzymes that dephosphorylate proteins that had been phosphorylated, and thereby activated, by  
25    the cyclic AMP system.

     The basis of approach (a) above is as follows. A system for the regulation of adenylate cyclase activity in plasma membranes consists of GTP; a  $G_i$  regulator protein which, when bound to GTP, inhibits the activity  
30    of adenylate cyclase; a  $G_s$  regulatory protein which, when bound to GTP, activates adenylate cyclase; and, agonists that increase the binding of GTP to  $G_i$  or  $G_s$ . It has now been discovered that agonists that increase

0 the binding of GTP to  $G_i$ , such as  $\alpha$ -adrenergic agents  
and adenosine A1 receptor agonists [e.g.,  
cyclopentyladenosine (CPA) and the (-) stereoisomer of  
N<sup>6</sup>-(phenylisopropyl)-adenosine (R-PIA)], particularly  
the latter agonists, are effective in reducing the  
5 resistance of brain endothelial cells. Such  
observations may be made in vitro in the blood-brain  
model of the invention or in vivo in brain infusion and  
behavioral test systems in mice, as shown in the in  
vivo examples below. For example, it was found that  $G_i$   
10 agonists that presumably inhibited the cellular  
production of cyclic AMP lowered the amount of morphine  
that had to be administered intravenously to mice in  
order to produce analgesia (morphine does not penetrate  
well into the brain). Another in vivo test system  
15 comprises the intravenous administration of a test drug  
to an unanesthetized animal, the injectate also  
containing labeled tracer substances that normally do  
not penetrate the BBB. Thereafter, the test animal is  
injected with an anesthetic, followed by phosphate-  
20 buffered saline and a tissue fixative. The brain is  
then removed and dissected, and the amount of tracer  
substance quantified. Observation with these test  
systems suggest that agents that inhibit adenylate  
cyclase and reduce cyclic AMP production increase the  
25 permeability of tight junctions and open up the blood-  
brain barrier, thereby providing a drug delivery  
system.

Approach (a) above also includes the use of  
inhibitors that block the binding to the receptors for  
30 the aforementioned  $G_s$  system of endogenous ligands,  
e.g., norepinephrine, that stimulate the  $G_s$  system. By  
this means, endogenous production of cyclic AMP is  
reduced, thereby reducing tight junction formation

0    between brain microvascular endothelial cells.  
Approach (a) also includes agents that directly inhibit  
adenylate cyclase, such as the synthetic nucleoside  
dideoxyadenosine.

5        The basis of approach (b) above is that  
competitive inhibitors of the action of cyclic AMP will  
increase the permeability of tight junctions, thereby  
opening up the blood-brain barrier. Compounds of this  
type that can be tested in the blood-brain model of the  
invention include the  $R_p$  diastereoisomer of cyclic AMP.

10       The basis of approach (c) above is as follows.  
Cyclic AMP is known to act physiologically by  
activating one or more protein kinases that, in turn,  
catalyze the phosphorylation of key proteins. Thus,  
inhibitors of protein kinases should nullify the  
15       effects of cyclic AMP on tight junction formation  
between brain endothelial cells. As will be detailed  
in the examples below, protein kinase inhibitors such  
as K252a and staurosporine at nM (10-200 nM)  
concentrations can markedly reduce the resistance of  
20       brain endothelial cell cultures. Both inhibitors were  
reversible. At the light microscope level, it was  
discovered that either removing cyclic AMP or adding a  
protein kinase inhibitor caused clear separations of  
endothelial cell tight junctions.

25       The basis of approach (d) above is that  
dephosphorylation of those key proteins whose  
phosphorylation had been catalyzed by cyclic AMP-  
activated protein kinases will produce an  
unphosphorylated protein that is inactive in  
30       maintaining tight junctions among endothelial cells.

0 Increased Cyclic GMP Concentrations or Activity

Cyclic GMP, another regulatory cyclic nucleotide, is produced from GTP by the enzyme guanylate cyclase.

It has now been discovered that increasing cyclic GMP concentrations or physiological activity in brain  
5 endothelial cells leads to a decrease in resistance, and thus to an increase in tight junction permeability. Increased concentrations or physiological activity of brain endothelial cell cyclic GMP can be achieved by, for example, 8-bromo-cyclic GMP, atrial natriuretic  
10 factor and sodium nitroprusside, and by cyclic GMP phosphodiesterase inhibitors such as dipyridamole (Research Biochemicals, Inc.) or Zaprinast (Rhone-Poulenc). It was observed, for example, that nitroprusside at concentrations of from 0.1 to 100  $\mu$ M  
15 markedly inhibited the effect of a cyclic AMP analogue, RO-20-1724, on elevating the resistance of brain endothelial cells in the blood-brain model of the invention. Such agents may be used in the aforementioned in vivo test systems to determine their  
20 effect on the opening up the blood brain barrier in test animals. For example, the in vivo morphine analgesia test system described below can be used to demonstrate that sodium nitroprusside opened up the blood-brain barrier to morphine, and that dipyridamole  
25 opened up the blood-brain barrier to enkephalin, an endogenous opiate in vertebrates that does not penetrate significantly into the brain when administered in the peripheral circulation.

30 Construction of a Chamber BBB Model

In a general embodiment of this invention, brain capillary endothelial cells are grown on a porous substratum-coated solid support, e.g., filters or

0 membranes. It has been found that endothelial cells  
can attach to and grow on Nucleopore polycarbonate  
filters (Costar, Inc., Cambridge, MA), Millicell CM and  
HA porous nitrocellulose filters (Millipore Corp,  
Bedford, MA), and collagen membranes (ICN Biomedical,  
5 Inc., Costa Mesa, CA). The Millicell CM and Nucleopore  
polycarbonate filters required pre-treatment, i.e.,  
coating, with extracellular matrix material (ECM, see  
below), components in order to promote adhesion of  
cells to the filter. Nucleopore filters promote media  
10 exchange across the filter, and permit cellular  
processes to cross through. Filters allow cells more  
completely to establish blood side and brain side  
domains, as they permit separate manipulation of the  
two compartments of the chamber.

15 Porous solid supports can be coated with ECM by  
soaking them in an aqueous solution of laminin,  
vitronectin, or fibronectin (typically, from about 10  
to about 50  $\mu\text{g/ml}$ ), Matrigel<sup>R</sup> (an extract of EHS  
sarcoma obtainable from Collaborative Res., Bedford,  
20 MA) in PBS, type I rat tail collagen or type IV  
collagen in dilute acetic acid (Collaborative Research,  
Inc., Collagen Corp, and New York Blood Bank, N.Y.), or  
astrocyte extracellular matrix (AECM).

In a preferred embodiment, filters were coated  
25 with astrocyte extracellular matrix (AECM) synthesized  
by astrocytes, in the following manner. Rat brain type  
I astrocytes, produced as described above, were grown  
on filters in a chemically-defined medium. Once cells  
reached confluence, they were lysed by a low ionic  
30 strength buffer containing a nonionic detergent, e.g.,  
Triton X-100, and rinsed with PBS containing a protease  
inhibitor such as aprotinin. This removed the cells  
and left behind AECM as a coating on the filter.

0 Alternatively, AECM was generated by solubilizing the  
cells of a confluent monolayer of astrocytes with  
nonionic detergent, and then extracting residual  
extracellular matrix components with a denaturant such  
as 6 M urea, 6 M guanidine HCl or 2 M  $MgCl_2$ . This  
5 extract was dialyzed against physiological saline  
before adding to endothelial cells being cultured on  
filters or used as a filter coat prior to adding  
endothelial cells.

In one embodiment of the chamber-type in vitro  
10 model of the BBB of this invention, an essentially  
confluent monolayer of rat brain type 1 astrocytes was  
disposed on one side of a porous solid support, and an  
essentially confluent monolayer of endothelial cells  
was disposed atop an ECM coating on the opposite side  
15 of the porous solid support. The thus-obtained  
co-culture device was then affixed in a chamber,  
effectively dividing the chamber into at least two  
compartments, one of which represents the brain side  
(endothelial cell side) and the other the blood side of  
20 a BBB. The cells were then placed in contact with a  
growth medium, preferably containing PDHS.

In another embodiment, an ECM-coated porous solid  
support was affixed in a chamber. On one ECM-coated  
surface of the porous solid support there was disposed  
25 a culture of astrocytes; endothelial cells were then  
plated at low density (about  $10^5$  cells/30 mm filter)  
onto the opposite ECM-coated surface. The astrocytes  
"condition" the growth medium that contacts the  
endothelial cells and may induce appropriate  
30 differentiative changes in the endothelial cells,  
including formation of tight junctions. The cells were  
grown in a culture medium containing serum, preferably  
PDHS.

0        In another embodiment, microvascular endothelial cells were disposed on an uncoated or EMC-coated porous solid support as described above, and the support was affixed in a chamber, on a surface of which there was disposed a culture of brain astrocytes. The growth  
5 medium must contact both the endothelial cells and astrocytes to effect biochemical interaction between the co-cultures.

      In yet another embodiment, microvascular endothelial cells were disposed on an ECM-coated porous  
10 solid support as described above, but astrocytes were absent from either the contralateral side of the porous solid support or from a surface of the chamber.

      Instead, the growth medium in the blood compartment of the chamber, i.e., the compartment opposite that which  
15 houses endothelial cells, was supplemented with from 0% to 100% with astrocyte-derived or endothelial cell-derived conditioned media, or with brain or other tissue extracts, obtained as described above, as required.

20        Agents that are intended to elevate intracellular concentrations of cyclic AMP in endothelial cells or to increase the concentration of effective cyclic AMP may be added to the growth medium, as can be dyes, e.g., trypan blue or Evans blue, or other macromolecules that  
25 are used to test for tight junction resistance.

      It is preferred that the glucose concentration of a growth medium in contact with endothelial cells not be higher than physiological in vertebrates, i.e., approximately 100 mg/dl.

### 30    Assay for Tight Junctions

      The presence of tight junctions in the endothelial layer of the BBB model can be detected using reagents



0 that recognize proteins associated with tight  
junctions. For example, the monoclonal antibody 40.76,  
made against ZO-1 tight junction protein, specifically  
recognizes an antigen on both bovine and mouse  
endothelial cells (Anderson, J.M. et al., J. Cell  
5 Biol., 106:1141 (1988); Stevenson, R.B., et al., J.  
Cell Biol., 103:755 (1986)). This approach allows the  
user to detect the formation of tight junctions among  
small subsets of endothelial cells, and to refine  
culture conditions to enhance the formation of tight  
10 junctions.

The degree of tightness of tight junctions can be  
also assessed by transcellular electrical resistance  
measurements. For transendothelial cell resistance  
measurements, cells were grown on a porous solid  
15 support, e.g., a filter or membrane attached to a  
holding device in order adequately to suspend the  
cellular monolayer, such as the Costar Transwell  
apparatus or the ICN Cellogen. Transmonolayer  
resistance is measured, for example, with the device of  
20 Perkins et al. (Perkins, F.M., et al., Am. J. Physiol.,  
241:C154 (1981)). Cells were maintained in a growth  
medium or physiological saline, and calomel electrodes  
on each of the endothelial cells are connected by a  
saturated KCl - 3% agar bridge. Current is passed  
25 between two Ag-AgCl electrodes and the voltage measured  
with a Keithly multimeter. Resistance is calculated  
from the change in voltage across the monolayer induced  
when a short current pulse (10-100  $\mu$ amp) is applied.  
The resistance of the filter or membrane alone is  
30 subtracted. The resistance, multiplied by the surface  
area of the filter or membrane, yields the resistance  
in ohms-cm<sup>2</sup>.

0       As noted above, peripheral binding of the toxin  
phalloidin reveals the presence of belt-like  
filamentous actin, a hallmark of tight junction  
formation among endothelial cells. Staining of  
filamentous actin by phalloidin can be visualized using  
5 derivatives such as phalloidin coumarin  
phenylisothiocyanate or fluorescent FITC-phalloidin or  
TRITC-phalloidin (Sigma Chem. Co., St. Louis, MO).

Another means for assessing the formation of tight  
junctions among endothelial cells is to determine the  
10 transport of macromolecules from the apical blood side  
to the abluminal brain side. For example, the water-  
soluble dye Evans blue (mol. wt. 960) that binds  
strongly to albumin (Freedman, F.B., et al., Am. J.  
Physiol., 216:675 (1969)), can be used to assess the  
15 tightness of newly formed endothelial cell junctions;  
tissues with tight junctions that exclude the dye or  
exhibit limited transport will remain white, whereas  
those without tight junctions or that exhibit  
significant transport capabilities will be stained blue  
20 as the dye passes through the junctions. Other water-  
soluble, macromolecular markers for tight junction  
formation include fluorescein isothiocyanate bound to  
dextran (FITC-dextran. mol. wt. 20,000, Sigma Chem.  
Co.) and <sup>125</sup>I-labeled albumin (DuPont/NEN, Wilmington,  
25 DE). Fluorescent dextrans of other sizes and sodium  
fluorescein itself may be used as well.

Still another means for assessing the tightness of  
junctions among endothelial cells in the blood-brain  
model of the invention is to compare the transport of a  
30 hydrophilic compound, e.g., sucrose, and a hydrophobic  
compound of similar size, e.g., chlorambucil, across  
filters with and without monolayered endothelial cells.  
When the transmonolayer resistance is high, the

0 transport of sucrose should be low compared to that of  
chlorambucil (or other hydrophobic compounds of similar  
size). Alternatively, when the resistance is high, the  
transport of sucrose should be much less (e.g., 50-fold  
or more) than across cell-free filters. Contrariwise,  
5 in "leaky" cell junctions, the relative transport of  
sucrose will be substantially increased.

Assessment of Ligand Binding, Transcytosis  
and Drug Delivery

The access in the model of the invention to both  
10 sides of a differentiated endothelium or ECM-coated  
porous solid supports permits the assay of specific  
binding and uptake of radiolabeled ligands from an  
apical (luminal) or basolateral (abluminal) aspect.  
Furthermore, by adding a labeled probe to one side of  
15 the porous solid support, one can assess the ability of  
the probe to be transcytosed from one side of the  
monolayer to the other.

The model also allows for testing the access of  
potential new therapeutics to the brain parenchyma.  
20 For example, drugs such as L-DOPA can cross the BBB,  
being recognized and transported by amino acid  
transporters. Lipophilic drugs are also able to  
penetrate the BBB. However, as indicated above,  
potentially therapeutic drugs that are not lipophilic  
25 and for which no specific transport mechanism exists  
may be unable to penetrate the BBB or may do so at  
rates insufficient to maintain a therapeutic drug level  
in the brain. The in vitro model of the BBB of this  
invention can also be used to test tight junction-  
30 disrupting compositions. It has been found by  
immunohistological methods that a molecule  
immunologically related to the mouse cell-adhesion

0 molecule E-cadherin is present on mouse endothelial cells. The expression of the E-cadherin-like molecule is enhanced in cultures of brain endothelial cells exhibiting increased resistance (Example 9).

#### Vasogenic Brain Edema

5 It is generally thought that brain edema is caused by increased tight junction permeability (decreased resistance) and/or increased pinocytosis. To the extent to which enhanced tight junction is important, agents that increase cyclic AMP concentration or  
10 physiological activity in brain microvascular endothelial cells may be of therapeutic value. These include cyclic AMP analogues, agonists that bind to receptors coupled to the Gs regulatory protein, adenylate cyclase activators, cyclic AMP-specific  
15 phosphodiesterase inhibitors, protein phosphatase inhibitors, and protein kinase stimulators. We have discovered, in this connection, that the phosphodiesterase primarily responsible for degrading cyclic AMP in brain microvascular endothelial cells is  
20 a member of the class termed the Type III cyclic GMP-noninhibitable phosphodiesterase, and that this enzyme is inhibited by compounds such as Rolipram and RO-20-1724 mentioned above.

#### Other Uses of the Model

25 While the foregoing illustrates preferred modes of practicing this invention, other embodiments of the basic concepts of the present invention can also be practiced. For example, the model can be used with cocultures of pulmonary or aortic arterial endothelial  
30 cells, with other cells of vascular walls, e.g., smooth muscle cells, in order to study morphological and

0 metabolic interactions between the two types of cells,  
as well as transcellular transport and drug  
permeability. The model may also be used as a  
chemotaxis chamber to study brain migration of  
lymphocytic cells through monolayers of brain  
5 endothelial cells to analyze CNS diseases such as  
multiple sclerosis. Yet another use of the model is to  
test other endothelial cell barriers, such as in the  
testes and retina. Still another use of this model is  
to screen reagents useful to prevent or ameliorate  
10 brain inflammation, such as in the embodiment described  
below wherein the model is used to screen compositions  
for the modulation of the adhesion of white blood cells  
to brain endothelia.

MODULATION OF LEUKOCYTE ADHESION  
TO BRAIN ENDOTHELIAL CELLS

15

The present blood brain barrier model was also  
used to screen reagents useful to prevent or ameliorate  
brain inflammation. The blood brain barrier model was  
used to select reagents that prevent the attachment of  
20 inflammatory white blood cells to the brain  
endothelium. This model, in conjunction with analysis  
of brain tissue, allowed identification of one of the  
receptors that white blood cells use to adhere to brain  
endothelial cells. Once this receptor was identified,  
25 reagents and methods useful for ameliorating or  
preventing inflammation were ascertained, as were  
therapeutic compositions useful for treating brain-  
inflammatory disease such as multiple sclerosis.

White blood cells (leukocytes) travel continuously  
30 in the general circulation. At the site of an injury  
or other inflammatory stimulus, cells that line blood  
vessels (endothelial cells) become activated to express

0 molecules that are adhesive for leukocytes. Thus,  
following an inflammatory stimulus, leukocytes bind to  
the activated endothelium. Once bound, the leukocytes  
migrate across the blood vessel wall to enter the  
injured site and release toxic mediators to combat  
5 infection. Unfortunately, the leukocyte toxins can also  
cause indiscriminate tissue damage. Such is the case  
with multiple sclerosis (MS). In MS, large numbers of  
leukocytes leave the blood stream within the brain and  
cause extensive tissue damage. See Hickey, W.F.,  
10 Psychoneuroimmunology II, Academic Press (1990).

In order for leukocytes to enter any tissue, they  
must first bind to the vascular endothelium. It has  
been shown in other disease systems that, in spite of  
the initial insult, if leukocyte binding to the  
15 endothelium at the damaged site is inhibited, then the  
leukocytes do not enter the tissue and further damage  
is greatly avoided. Simpson et al., J.Clin.Invest. 81:  
624-629 (1988) disclose that the administration of a  
monoclonal antibody that binds to a leukocyte cell  
20 adhesion-promoting glycoprotein (Mol; CD11b/CD18)  
resulted in reduced injury to heart tissue because  
fewer leukocytes (neutrophils) bound to the heart  
tissue.

The mechanics of leukocyte adhesion to endothelial  
25 cells involves, in part, the binding of cell surface  
receptors on leukocytes to the corresponding cell  
surface receptors on endothelia. Both leukocytes and  
endothelial cells are known to express various  
adhesion-promoting receptors at various times in  
30 response to various stimuli. For reviews of adhesion  
receptors of the immune system, see generally,  
Springer, Nature 346: 425 -434 (1990), and Osborn, Cell  
62: 3-6 (1990) both of which are herein incorporated by

0 reference. The expression of cell adhesion molecules is  
not predictable, and may vary widely in response to  
different inflammatory stimuli and in different  
anatomical locations. For example, Tuomanen et al., J.  
Exp. Med. 170: 959-968 (1989) show that antibodies  
5 directed against the CD18 family of adhesion-promoting  
receptors blocks the migration of leukocytes across the  
blood brain barrier in response to acute inflammatory  
stimulus of bacterial origin. Anti-CD18 was shown to  
not block leukocyte migration to the lung. Vedder et  
10 al., Surgery 106: 509 (1989).

Circulating leukocytes may express the VLA-4  
receptor, and this has been show to bind to the VCAM-1  
receptor on cytokine-activated human endothelial cells.  
Elices, et al., Cell 60: 577-584 (1990). The different  
15 types of molecules induced on blood brain barrier  
endothelial cells during brain inflammation, and the  
role that they play in chronic inflammatory brain  
disease, such as MS, is poorly understood.

A. Ameliorating or Preventing Brain Inflammation

20 The present embodiment was directed toward finding  
reagents that modulate leukocyte adhesion in the brain.  
The blood brain barrier model of the present invention  
was one system used. Using this model, brain  
endothelial cell samples, prepared in accordance with  
25 the present description, were activated with  
inflammation mediators. To a panel of these activated  
cell samples, leukocytes were introduced in the  
presence of a different putative receptor-blocker for  
each sample. Individual samples were assayed for the  
30 presence or degree of leukocyte adhesion. Here, among  
the various reagents tested, two antibodies directed  
against VLA-4 (the leukocyte cell adhesion molecule)

0 were shown to block lymphocyte binding to brain  
endothelium.

A separate assay produced the same results. Essentially, slices of brain tissue were analyzed for their ability to bind leukocytes in the presence of  
5 putative cell adhesion modulators. In this system, another novel aspect of the present invention was developed. Rats were injected with human tumor cells in order to induce inflammation in the brain. Previously, it was not known that this method could induce traffic  
10 across the blood brain barrier into the brain. Further, the type of inflammation induced is very much like that seen in multiple sclerosis victims where inflammation is characterized by small vessels, with activated almost cuboidal endothelium. The vessels appear very  
15 similar to the "high endothelial venules" seen in lymphoid tissues. Further, the vessels are surrounded by a cusp of lymphocytes, and active lymphocyte traffic is apparent. While MS-type inflammation has been observed, it has never before been induced by this  
20 method. Thus, induction of brain inflammation using tumor cells possesses great utility in obtaining tissue for an in vitro model of multiple sclerosis.

After an appropriate length of time, rat brains in which inflammation had been induced were removed and  
25 sectioned. To these sections, leukocytes were added, in the presence of the putative cell adhesion modulator to be screened. Here too it was found that the anti-VLA-4 antibodies inhibited leukocyte adhesion.

This inhibition is graphically illustrated in the  
30 Figures. Figure 8, Panel A shows a brain section in which no antibody is added. The small dark dots are leukocytes against the background of inflamed brain endothelial cells. As can be seen, the leukocytes are



0 quite densely bound to the vessels in inflamed tissue.  
Figure 8, Panel B shows inhibition of binding by  
antibodies directed against the  $\beta 1$  subunit of VLA-4.  
Figure 9 shows a brain endothelial culture to which  
lymphocytes have bound. Panel A illustrates binding to  
5 unstimulated endothelium. Panel B shows binding to  
endothelium stimulated for twelve hours by  $\text{TNF}\alpha$ . In  
Panel C, the lymphocytes have been pretreated with  
anti- $\beta$ -1 integrin and their binding to stimulated  
endothelium is greatly inhibited. As described in  
10 Example 21, below, the binding density of human  
leukocytes to brain sections was confirmed by using an  
internal population of mouse leukocytes, a population  
not recognized by the anti-human reagent. This  
quantification confirmed the visual observation that  
15 anti-VLA antibodies prevented leukocyte binding to  
brain sections in which multiple sclerotic-type  
inflammation had been induced. (Figure 10). Further,  
leukocyte binding to cultured endothelium was  
quantified by prelabeling the cells with  $^{125}\text{I}$ ; the  
20 inhibitory effects of anti- $\beta$  are illustrated in  
Figure 11.

Different cell adhesion molecules are expressed in  
different tissues in response to a variety of stimuli.  
Brain-specificity may be beneficial in administering a  
25 leukocyte adhesion modulator for therapeutic purposes.  
The VLA-4 leukocyte adhesion molecule is known to be  
expressed throughout the body under various conditions.  
Other tissues, apart from brain tissue, were analyzed  
in order to determine if either the anti- $\alpha$ -4 or the  
30 anti- $\beta$ -1 antibody had any immune reaction in those  
tissues. As shown in more detail in Example 22, the  
anti- $\alpha$ -4 inhibited lymphocyte binding to normal  
intestinal lymphoid tissue, but did not affect binding

0 to normal lymph nodes. The anti- $\beta$ -1 antibody does not inhibit binding to lymph nodes and would not be expected to affect binding to intestinal lymphoid tissue.

VLA-4 is a member of the  $\beta$ 1 integrin family of  
5 cell adhesion molecules, each of which is comprised of two subunits, an  $\alpha$  and a  $\beta$  chain. There are at least six  $\beta$ 1 integrins, all sharing the same  $\beta$ 1 chain and each having a distinct  $\alpha$  chain. These six receptors all bind a different complement of the various cell  
10 matrix molecules, such as fibronectin, laminin, and collagen. VLA-4, for example, binds to fibronectin. VLA-4, however, is unique in that it also binds to a non-matrix molecule that is expressed by endothelial  
15 cells. This molecule is called VCAM-1, and is thought to be expressed on endothelium at various geographic locations in response to various stimuli. Distinct epitopes of VLA-4 are responsible for the fibronectin and VCAM-1 binding activities, and each activity can be inhibited independently.

20 One of the monoclonal antibodies presently used, HP2/1 reacts with the  $\alpha$  chain of VLA-4 and blocks only its binding to VCAM-1. It does not affect binding of VLA-4 to fibronectin, nor does it affect the activity of the other members of the  $\beta$ 1 integrin family.

25 However, the  $\alpha$  chain of VLA-4 also interacts with a distinct  $\beta$  chain, called  $\beta$ p. This receptor mediates all lymphocyte binding to intestinal lymphoid tissues.

Another of the antibodies used, the monoclonal antibody, HP2/1, reacting with VLA-4 $\alpha$  blocks the  
30 activity of this molecule, i.e., it prevents the binding of VLA4 $\alpha\beta$ p to intestinal endothelium (illustrated in Table 12). The monoclonal antibody, AIIB2 reacts with the  $\beta$ 1 chain that is common to all

0 members of the  $\beta 1$  integrins and potentially  
immunoreacts with the entire family, including the  
fibronectin and the VCAM-1 binding activities of VLA-4.  
It would not be expected to inhibit lymphocyte binding  
to intestinal endothelium, however, because it would  
5 not bind to  $\beta p$ .

Reagents which selectively react against the VLA-  
4/VCAM-1 target are also envisioned. For example, an  
antibody which interacts with the VCAM-1 binding domain  
of VLA4 $\alpha$  in conjunction with the  $\beta 1$  chain would block  
10 only lymphocyte migration into sites of inflammation,  
such as the brain during multiple sclerosis. This  
reagent further would not affect matrix interactions  
(mediated by all members of the  $\beta 1$  integrins) nor would  
it affect normal intestinal immunity (mediated by VLA-  
15 4 $\alpha\beta p$ ). The production of this, and other such reagents  
is well within the skill of the art.

B. VLA-4/VCAM-1 Directed Cell  
Adhesion Modulators And Uses

The VLA-4/VCAM-1 molecules, instrumental in brain  
20 inflammation (particularly MS-type brain inflammation)  
provide molecular targets that can be put to a variety  
of uses. The present invention thus encompasses these  
uses and related compositions.

First, as is shown by Example 23, receptors  
25 against the VLA-4 ligand may be used to modulate  
leukocyte adhesion to brain endothelial cells. Herein,  
the term "receptor" is used to denote a biologically  
active molecule that binds to a ligand. For example,  
antibodies or fragments thereof, which immunoreact with  
30 the VLA-4 molecule may be useful to prevent leukocyte  
binding to brain endothelial cells. Peptides, or  
peptidomimetics or related compounds, which can act to  
bind the cellular adhesion molecule, are also

0 contemplated, and these may be made synthetically by  
methods known in the art. Other receptors which react  
with a VLA-4 ligand will be apparent to those skilled  
in the art.

5 Additionally, receptors against a VCAM-1 ligand  
may be used to modulate leukocyte adhesion to brain  
endothelial cells. Either way, one cell adhesion  
molecule is blocked, and one pathway of leukocyte  
adhesion is terminated.

10 It should be recognized that for therapeutic  
purposes, therapeutically effective compositions for  
preventing or ameliorating brain inflammation  
containing such VLA-4 or VCAM-1 directed receptors are  
contemplated as within the scope of the present  
invention. For example, therapeutic compositions  
15 including at least one VLA-4 receptor or VCAM-1  
receptor as well as other therapeutic compositions  
could be used to prevent or ameliorate inflammation of  
brain endothelial cells. Another example is the use of  
a VCAM-1 receptor, to which is attached a drug useful  
20 for treating MS or other inflammatory condition, for a  
drug delivery vehicle which also prevents the adhesion  
of leukocytes to the VCAM-1 molecule. Peptides or  
peptidomimetics or other molecules, which serve to  
substantially mimic one cell adhesion molecule or the  
25 other, could be used in competition therapy wherein  
such peptides or peptidomimetics (or other compounds)  
compete for the available locations on the surface of  
either the leukocyte (if substantially mimicking VCAM-  
1) or the endothelial cell (if substantially mimicking  
30 VLA-4).

Suitable pharmaceutical carriers and their  
formulations are described in Martin, Remington's  
Pharmaceutical Sciences, 15th Ed. (Mack Publishing Co.,

0 Easton 1975). Such compositions will, in general,  
contain an effective amount of the active compound  
together with a suitable amount of carrier so as to  
prepare the proper dosage form for proper  
administration to the host. Useful pharmaceutical  
5 carriers for the preparation of the pharmaceutical  
compositions hereof can be solids, liquids or gases;  
thus, the compositions can take the form of tablets,  
pills, capsules, powders, enterically coated or other  
protected formulations (such as by binding on ion  
10 exchange resins or other carriers, or packaging in  
lipid protein vesicles or adding additional terminal  
amino acids), sustained release formulations, solutions  
(e.g., ophthalmic drops), suspensions, elixirs,  
aerosols, and the like. Water, saline, aqueous  
15 dextrose, and glycols are preferred liquid carriers,  
particularly (when isotonic) for injectable solutions.  
The carrier can be selected from various oils including  
those of petroleum, animal, vegetable or synthetic  
origin, for example, peanut oil, soybean oil, mineral  
20 oil, sesame oil, and the like. Suitable pharmaceutical  
excipients include starch, cellulose, talc, glucose,  
lactose, sucrose, gelatin, malt, rice, flour, chalk,  
silica gel, magnesium stearate, sodium stearate,  
glycerol monostearate, sodium chloride, dried skim  
25 milk, glycerol, propylene glycol, water, ethanol, and  
the like. The compositions may be subjected to conven-  
tional pharmaceutical expedients such as sterilization  
and may contain conventional pharmaceutical additives  
such as preservatives, stabilizing agents, wetting or  
30 emulsifying agents, salts for adjusting osmotic  
pressure, buffers, and the like.

In the practice of the therapeutic methods of the  
present invention, an effective amount of the active

0 compound, including derivatives or salts thereof, or a  
pharmaceutical composition containing the same, as  
described above, is administered via any of the usual  
and acceptable methods known in the art, either singly  
or in combination with another compound or compounds of  
5 the present invention or other pharmaceutical agents  
such as anti-inflammatory agents, or other therapeutics  
known to have an effect on inflammation or the like.  
These compounds or compositions can thus be adminis-  
tered orally, sublingually, topically (e.g., on the  
10 skin or in the eyes), parenterally (e.g.,  
intramuscularly, intravenously, subcutaneously or  
intradermally), or by inhalation, and in the form of  
either solid, liquid or gaseous dosage including  
tablets, suspensions, and aerosols, as is discussed in  
15 more detail above. The administration can be conducted  
in single unit dosage form with continuous therapy or  
in single dose therapy ad libitum.

In one preferred embodiment, the therapeutic  
methods of the present invention are practiced when the  
20 relief of symptoms is specifically required or perhaps  
imminent; in another preferred embodiment, the method  
hereof is effectively practiced as continuous or  
prophylactic treatment.

In the practice of the therapeutic methods of the  
25 invention, the particular dosage of pharmaceutical  
composition to be administered to the subject will  
depend on a variety of considerations including the  
nature of the disease, the severity thereof, the  
schedule of administration, the age and physical  
30 characteristics of the subject, and so forth. Proper  
dosages may be established using clinical approaches  
familiar to the medicinal arts. It is presently  
believed that dosages in the range of 0.1 to 100 mg of

0 compound per kilogram of subject body weight will be  
useful, and a range of 1 to 100 mg per kg generally  
preferred, where administration is by injection or  
ingestion. Topical dosages may utilize formulations  
containing generally as low as 0.1 mg of compound per  
5 ml of liquid carrier or excipient, with multiple daily  
applications being appropriate.

Imaging reagents are also contemplated. A tracer  
molecule, detectable in radiographic or other imaging  
techniques) could be linked to an anti-VCAM or anti-  
10 VLA-4 reagent to identify areas of active leukocyte  
traffic in the brain. This is useful in diagnostic  
protocols and in determining the progression of the  
disease or the effectiveness of therapy, for example.

Other uses, formulations, compositions, and  
15 processes will be readily apparent to those skilled in  
the art.

The following examples are illustrative of several  
embodiments of this invention, and should not be  
construed as in any way limiting the invention as  
20 recited in the claims.

#### EXAMPLE 1

##### ELECTRICAL RESISTANCE OF ENDOTHELIAL CELL CULTURES TREATED WITH CYCLIC AMP

Bovine brain capillary endothelial cells were  
25 grown on polycarbonate filters in a conditioned growth  
medium containing either 5% or 10% PDHS. In controls,  
the growth medium contained 5% PDHS ( $\square$ ) or 10% PDHS  
( $\blacksquare$ ). In experimental cultures, the growth medium was  
supplemented with either 5% PDHS + 250  $\mu$ M 8-(4-  
30 chlorophenylthio) cyclic AMP ( $\bigcirc$ ) or 10% PDHS + 250  $\mu$ M  
8-(4-chlorophenylthio)cyclic AMP + 35  $\mu$ M RO-20-1724, a  
cyclic AMP phosphodiesterase inhibitor ( $\bullet$ ).

0 Transmonolayer electrical resistances were then determined; these are shown in Fig. 1.

The cyclic AMP analogue alone greatly increased transmonolayer electrical resistance, which is indicative of tight junction formation. Resistances of  
5 about 400 ohm-cm<sup>2</sup>, were obtained with monolayers treated with both the cyclic AMP analogue and an agent (RO-20-1724) that inhibited degradation of cyclic AMP.

#### EXAMPLE 2

##### 10 TRANSPORT OF LABELED ALBUMIN ACROSS ENDOTHELIAL CELLS AS A FUNCTION OF TRANSMONOLAYER ELECTRICAL RESISTANCE

The flux of <sup>125</sup>I-labeled albumin across monolayers of primary cultures of bovine retina endothelial cells and of Madin-Darby Canine Kidney epithelial cells  
15 disposed on 0.4 μ pore-sized polycarbonate filters was determined as a function of tight junctions as reflected in trans-monolayer electrical resistance of each cell type. The results of such experiments are plotted in Fig. 2. The height of the bars in the  
20 histogram is a reflection of leakage of albumin through the junctions between cells - the higher the bar, the greater the leakage of albumin.

Control cell-free filter, of course, exhibited the smallest impediment to albumin flux.

25 Substantial flux of albumin across monolayers of bovine retina cells occurred when the electrical resistance was only 20 ohm-cm<sup>2</sup>, and this flux was reduced by 75% in cultures exhibiting an electrical resistance of 60 ohm-cm<sup>2</sup>.

30 In contrast, the flux of albumin was virtually abolished in monolayers of MDCK cells in which



transmonolayer electrical resistances of greater than 200 ohm-cm<sup>2</sup> were observed.

### EXAMPLE 3

#### EFFECTS OF VARIOUS AGENTS ON TRANSENDOTHELIAL CELL ELECTRICAL RESISTANCE

Bovine brain capillary endothelial cells were grown to substantial confluency on either 3  $\mu$ m polycarbonate or 0.4  $\mu$ m nitrocellulose filters.

Cultures were either untreated for 24 hours (control) or grown for 24 hours in a growth medium containing either 250  $\mu$ M 8-(4-chlorophenylthio) cyclic AMP + 35  $\mu$ M RO-20-1724 (cAMP) or these two agents plus 50% (w/v) bovine aortic endothelial cell-derived conditioned medium (cAMP - BAEC-CM). Transmonolayer electrical resistances were then determined. The results are shown in Table 1.

Table 1

<u>Treatment</u>	<u>Filter</u>	<u>Resistance (ohm-cm<sup>2</sup>)</u>
Control	3 $\mu$ m polycarbonate	38
cAMP	3 $\mu$ m polycarbonate	205
cAMP + BAEC-CM	3 $\mu$ m polycarbonate	348
Control	0.4 $\mu$ m nitrocellulose	94
cAMP + BAEC-CM	0.4 $\mu$ m nitrocellulose	320

The results indicate that cAMP alone produced about a 6-fold increase in transmonolayer resistance, to about 200 ohm-cm<sup>2</sup>. The combination of BAEC-CM and cAMP increased resistance by about 10-fold, to about 350 ohm-cm<sup>2</sup>, on polycarbonate filters.

0

## EXAMPLE 4

EFFECTS OF ELEVATION OF  
INTRACELLULAR CYCLIC AMP CONCENTRATION ON  
TRANSENDOTHELIAL CELL ELECTRICAL RESISTANCE

5 Bovine brain capillary endothelial cells were grown essentially to confluency on 0.4  $\mu\text{m}$  polycarbonate filters.

Cultures were either untreated for 24 hours (Control) or treated for 24 hours with 250  $\mu\text{M}$  8-(4-chlorophenylthio) cyclic AMP (cAMP), 10  $\mu\text{M}$  isoproterenol (ISO), 10  $\mu\text{M}$  5-hydroxytryptamine (5-HT), or 10  $\mu\text{M}$  forskolin. In all cultures, 35  $\mu\text{M}$  RO-20-1724 was also present. Transmonolayer electrical resistances were then determined. Each value in Table 2 represents the average of 3 replicate experiments, referred to control cells to which was assigned a resistance value of 100.

Table 2

	<u>Treatment</u>	<u>Relative Resistance</u>
20	Control	100
	cAMP	465
	ISO	439
	5-HT	586
	Forskolin	834

25 The results indicated that any agent that elevated effective intracellular cyclic AMP concentrations enhanced transmonolayer electrical resistance by at least 4-fold. The greatest enhancement of resistance (over 8-fold) was obtained by the use of forskolin, a compound that activates adenylate cyclase directly.

30

0

## EXAMPLE 5

EFFECTS OF CYCLIC AMP, CONDITIONED MEDIUM  
AND ASTROCYTE EXTRACELLULAR MATRIX ON  
TRANSENDOTHELIAL CELL ELECTRICAL RESISTANCE

Bovine brain capillary endothelial cells were  
5 grown to essential confluency on nitrocellulose or  
polycarbonate filters. In A and B, the filters were  
first coated with type I collagen and fibronectin. In  
C and D, type I astrocytes from neonatal rat brain were  
then grown to confluency on these collagen and  
10 fibronectin-coated filters. In case C, the astrocytes  
were lysed in 1% Triton X-100 in 5 mM Tris buffer, pH  
7.5, for 30 min. at 25°C. The filters were rinsed  
again in PBS. In case D, astrocytes were grown as in  
case C, but then treated with 5 mM EDTA in PBS for 30  
15 min. at 37°C to remove the cells. These filters were  
also treated with 25 mM  $\text{NH}_4\text{OH}$  and rinsed in PBS.  
Bovine brain endothelial cells were grown to confluency  
on these different types of filters. In B, C and D,  
cells were treated as in Table 1 with 250  $\mu\text{M}$  8-(4-  
20 chlorophenylthio) cyclic AMP (cAMP), 35  $\mu\text{M}$  35  $\mu\text{M}$   
RO-20-1724 and bovine endothelial cell conditioned  
medium (BAEC-CM). Transendothelial electrical  
resistances were determined; resistances were  
normalized to a control value of 100 in Table 3.  
25 The combination of the cyclic AMP derivative,  
phosphodiesterase inhibitor and BAEC-CM produced a  
substantial increase in transmonolayer electrical  
resistance. These effects were further potentiated  
when the cells were grown on astrocyte extracellular  
30 matrix prepared as described in C. When cells were  
grown on astrocyte extracellular matrix prepared as in  
D, they did not grow to confluency (and, hence, the  
transmonolayer resistance was low). When cells were

- 0 grown on matrices prepared from other cell types (such as endothelial cells), there was no increase in resistance.

Table 3

	<u>Treatment</u>	<u>Relative Resistance</u>
5	A) Control	100
	B) cAMP + BAEC-CM	980
	C) cAMP + BAEC-CM + astro-ECM #1	2652
	D) cAMP + BAEC-CM + astro-ECM #2	138

EXAMPLE 6

10 EFFECT OF ASTROCYTE-DERIVED CONDITIONED MEDIUM  
ON ELECTRICAL RESISTANCE OF BRAIN ENDOTHELIAL CELLS

- Freshly-dissociated bovine brain endothelial cells (BBEC) were plated on tissue culture dishes either in the absence or presence of astrocyte-derived  
15 conditioned medium (ADCM). Cells were then plated onto collagen-fibronectin-coated filters, again in the absence or presence of ADCM. After the cells reached confluency, samples were treated with 250  $\mu$ M 8-(4-chlorophenylthio) cyclic AMP and 35  $\mu$ M RO-20-1724  
20 (+cAMP in Table 4).

The data of Table 4 demonstrate that the highest resistances were obtained when cells were placed initially in ADCM.

0

Table 4

	<u>Growth Medium*</u>	<u>Plating Medium</u>	<u>Electrical resistance</u>	
			<u>- CAMP</u>	<u>+ CAMP</u>
			ohm-cm <sup>2</sup>	
	MEM/FCS	MEM/FCS	11	42
5	50% MEM/FCS: 50% ADCM	MEM/FCS	12	142
	MEM/FCS	50% MEM/FCS: 50% ADCM	27	160
10	50% MEM/FCS: 50% ADCM	50% MEM/FCS: 50% ADCM	46	312

\*MEM, minimal essential medium; FCS, fetal calf serum; ADCM, astrocyte-derived conditioned medium.

## EXAMPLE 7

15

EFFECT OF FETAL CALF SERUM REDUCTION ON  
ELECTRICAL RESISTANCE OF BRAIN ENDOTHELIAL CELLS

Bovine brain endothelial cells were dissociated and plated on tissue culture dishes in 50% MEM/FCS - 50% ADCM. Cells were then passaged onto collagen-fibronectin-coated filters and grown in the media indicated in Table 5. Conditions were as in Example 6, except that some cells were grown in 50% serum-free defined medium (N2). The results are shown in Table 5.

The data demonstrate that the highest resistance were attained with cells grown in ADCM and cyclic AMP analogues in the presence of reduced fetal calf serum. The same result was obtained when the defined medium N2 was replaced by MEM.

0

Table 5

	<u>Treatment of cells*</u>	<u>Resistance</u> ohm-cm <sup>2</sup>
	MEM/FCS	40 ± 6
	+ cAMP	163 ± 22
5	+ ADCM	56 ± 15
	+ cAMP + ADCM	345 ± 64
	50% MEM/FCS, 50% N2	61 ± 2
	+ cAMP	305 ± 50
	+ ADCM	115 ± 11
10	+ cAMP + ADCM	625 ± 82

\* MEM, minimal essential medium; cAMP, cyclic AMP;  
ADCM, astrocyte-derived conditioned medium.

## EXAMPLE 8

15

TRANSPORT DATA ACROSS HIGH RESISTANCE MONOLAYERS

Figure 3 shows transport data across high resistance monolayers of bovine endothelial cells on filters in the blood-brain model of the invention, using labeled sucrose (360 dalton, hydrophilic) and  
20 labeled chlorambucil (304 dalton, mildly hydrophobic anti-tumor compound).

Although these compounds are of similar size, the hydrophobic compound was transported much better than was the hydrophilic compound across filters with cells,  
25 compared to transport rate across cell-free filters.

Sucrose was almost entirely prevented from leaking between the cells. This is a significant advance over previous models in which the difference in the rate of sucrose across filters with and without cells was 3 to  
30 5-fold due to leaky tight junctions in those models.

0

## EXAMPLE 9

EFFECTS OF PROTEIN KINASE INHIBITORS ON  
RESISTANCE OF BRAIN ENDOTHELIAL CELLS

Bovine brain endothelial cells were grown to confluency on filters in the blood-brain model of the invention as described in Examples 1, 3 and 5 above, and the effects of protein kinase inhibitors K252a and staurosporine on resistance determined. These compounds are non-specific in that they inhibit protein kinases A and C, MLCK, etc. The results are shown in Table 6.

Table 6

<u>Drug</u>	<u>Resistance (% of control)</u>		
	<u>1 hr.</u>	<u>2.5 hr.</u>	<u>24 hr.</u>
K252a (200 nM)	79	25	24
Staurosporine			
10 nM	108	20	5.2
30 nM	64	7.7	2.3
100 nM	5.5	0	0

Both compounds, but particularly staurosporine, were remarkably effective in reducing resistance, i.e., opening up tight junctions. The effects of both inhibitors were reversible.

At the light microscope level, either removing cyclic AMP or adding a protein kinase inhibitor caused clear separation of endothelial cell tight junctions.

## EXAMPLE 10

EFFECT OF PROTEIN KINASE INHIBITION  
ON BRAIN UPTAKE IN VIVO

Staurosporine was administered by intracarotid infusion. The transport of <sup>3</sup>H-sucrose and <sup>125</sup>I-BSA was

0 then determined. The data in Table 7 represent the amount of radioactivity remaining in the brain after infusion and washout. An average of 3-4 animals was used in each category.

Table 7

5	<u>Experiment 1 (DPM)</u>		
		<u><sup>3</sup>H-Sucrose</u>	<u><sup>125</sup>I-BSA</u>
	Saline control	$6.2 \times 10^3$	178
	Staurosporine	$24.3 \times 10^3$	835
	<u>Experiment 2 (DPM)</u>		
10		<u><sup>3</sup>H-Sucrose</u>	<u><sup>125</sup>I-BSA</u>
	Saline control	$1.4 \times 10^3$	98
	Staurosphorine	$8.7 \times 10^3$	520

The results showed that staurosporine enhanced  
15 entry into the brain of both small (sucrose) and large (BSA) molecules, as the result of the inhibitan-  
ce of the activity of cyclic AMP-activated protein kinase.

#### EXAMPLE 11

20 EFFECT OF G<sub>i</sub> RECEPTOR AGONISTS AND AGENTS  
THAT INCREASE CYCLIC GMP ON BLOOD-BRAIN BARRIER  
IN AN IN VIVO TEST SYSTEM

The test compound was delivered to a restrained, unanesthetized mouse (30-35g) via the tail vein. The injectate also contained 10  $\mu$ Ci <sup>3</sup>H-sucrose and 1  $\mu$ Ci  
25 <sup>125</sup>I-BSA as tracer substances that do not normally penetrate the BBB in vivo. Fifteen or sixty minutes after the injection, the animal, anesthetized with 2% Avertin, was perfused via venipuncture with phosphate buffered saline followed by fixative. The brain was  
30 removed immediately, the medulla, pons and hypothalamus



0 excised and the remaining tissue homogenized by  
 passage through a 3 cc needle into a preweighed  
 scintillation vial containing 1.5 ml Soluene (Packard).  
 The vial was weighed to determine wet tissue weight.  
 Ten ml of Inst-Gel (Packard) was added to each sample  
 5 after overnight incubation at 75°C. The samples were  
 counted for DPM by liquid scintillation spectrometry.  
 Values are expressed as DPM/gm tissue. A minimum of  
 four animals/group were used for each experiment. Data  
 are expressed as the mean fold-increase which is  
 10 derived by dividing the treatment value by the control  
 value. Numbers in parenthesis represent the number of  
 experiments carried out for the particular condition.

Table 8

15	<u>Compound</u>	<u>Radioactivity (DPM)</u>			
		<u><sup>3</sup>H-sucrose</u>		<u><sup>125</sup>I-BSA</u>	
	CPA, 100 ug/kg	15'	1.6 (2)	1.2	(2)
		60'	2.6 (2)	1.9	(2)
	R-PIA, 25 ug/kg	15'	2.0 (1)	1.2	(1)
		60'	3.0 (2)	1.8	(2)
20	Nitroprusside, 3 mg/kg	15'	1.5 (1)	1.0	(1)
		60'	2.3 (2)	1.2	(2)

25 The results show that adenosine Gi receptor  
 agonists (such as cyclopentyladenosine, CPA) and the  
 (-) stereoisomers of N<sub>6</sub>-(phenylisopropyl)-adenosine  
 (R-PIA) increased the uptake by brain of small  
 (sucrose) and large (BSA) molecules by as much as 300%.

0

## EXAMPLE 12

EFFECT OF TIGHT JUNCTION PERMEABILITY MODULATORS  
ON BEHAVIORAL ASSAY

The behavioral assay is designed to demonstrate delivery of a drug into the brain parenchyma at levels  
5 sufficient to have a therapeutic effect. Morphine and the naturally occurring opioid peptides, endorphin and enkephalin, bind to  $\mu$  opioid receptors in the brain and suppress the sensation of pain. This analgesic effect can be demonstrated with mice in the hot plate assay.  
10 Mice are placed on a surface uniformly heated to 55°C. The time it takes for the mouse to respond to the heat stimulus by licking its front or hind paws is measured. Morphine (700 MW) delivered by i.v. injection at doses of 1-10 mg/kg, has an analgesic effect in that it  
15 increases the latency of response to the heat stimulus measured 15 minutes after the injection. The latency is expressed as % analgesia.

20 
$$\% \text{ analgesia} = \frac{(t_e - t_c) \times 100}{(T - t_c)}$$

$t_e$  = experimental latency at given dose of analgesic  
 $t_c$  = control latency with no analgesic  
 $T$  = 60 sec., the maximum allowed latency

25 The purpose of these experiments is to test the ability of putative BBB openers to shift the morphine dose response curve to lower doses and thus have analgesic activity when delivered peripherally. In this experiment (Fig. 4) 25  $\mu$ g/kg of CPA potentiated the  
30 effects of morphine, particularly at lower morphine levels. ●, morphine alone; x, CPA + morphine

Thus, CPA, which reduces cyclic AMP production, lowered the amount of morphine that had to be administered to produce analgesia, i.e., CPA opened the  
35 blood-brain barrier.

0

## EXAMPLE 13

INHIBITION BY NITROPRUSSIDE OF EFFECT OF CYCLIC AMP  
ON ENDOTHELIAL CELL TIGHT JUNCTIONS

Confluent layers on filters of bovine brain  
endothelial cells not previously treated with cyclic  
5 AMP analogues were stimulated with RO-20-1724 (+ RO in  
Fig. 5) at the beginning of the experiment so as to  
increase cyclic AMP levels and resistance or left  
untreated (- RO). Other cultures were treated with  
RO-20-1724 plus sodium nitroprusside (NitroP) in  
10 various concentrations. Nitroprusside is known to  
increase the activity of the Gi system. The resistance  
of the cells was then determined as above.

The results, shown in Fig. 5, show that  
nitroprusside inhibited the increase in resistance  
15 produced by cyclic AMP elevation in a dose-dependent  
manner.

## EXAMPLE 14

EFFECT OF SODIUM NITROPRUSSIDE ON  
MORPHINE-INDUCED ANALGESIA

20 The effects of sodium nitroprusside, an agent that  
increases cyclic GMP levels, on morphine-induced  
analgesia in mice were determined by the assay system  
of Example 12, and are shown in Fig. 6. Nitroprusside  
(+ NP) decreased the amount of morphine required to  
25 produce analgesia, suggesting that the former opened up  
the blood-brain barrier to the latter.

## EXAMPLE 15

APPEARANCE OF E-CADHERIN ON ENDOTHELIAL CELLS

30 Bovine brain endothelial cells were grown to  
confluency on filters either in control medium (low  
resistance cultures) or under conditions of enhanced

0 cyclic AMP plus endothelial cell conditioned medium as  
in Examples 1, 3 and 5 (high resistance cultures).  
After transendothelial cell resistance measurements,  
cultures were fixed and labeled with a rabbit antibody  
prepared against mouse E-cadherin with a fluorescent  
5 FITC-conjugate of goat anti-rabbit immunoglobulin. The  
high resistance cultures stained much more brightly for  
E-cadherin than did low resistance cultures, and also  
showed some localization of the E-cadherin around cell  
borders. As E-cadherin appears to be expressed  
10 specifically in endothelial cells in brain, these  
observations are a further demonstration that  
treatments that increase electrical resistance of brain  
endothelial cells in culture also cause them to adopt  
another characteristic property of the BBB.

15

## EXAMPLE 16

EFFECT OF INHIBITION OF CYCLIC GMPPHOSPHODIESTERASE ON ENKEPHALIN-INDUCED ANALGESIA

The behavioral assay described in Example 12,  
modified to induce analgesia with an endogenous opiate,  
20 enkephalin (20 mg/kg), rather than with morphine, was  
applied to mice treated with the cyclic GMP  
phosphodiesterase inhibitor, dipyridamole. The results  
are shown in Table 9.

0

Table 9

<u>Experiment</u>	<u>Percent Analgesia</u>	
	<u>Enkephalin</u>	<u>Enkephalin + Dipyridamole</u>
1	2.8	13.5
2	6.4	25.8

5

These results are consistent with the theory that elevation of intracellular levels of cyclic GMP increase the permeability of brain microvascular endothelial cell tight junctions to morphine.

10

EXAMPLE 17

EFFECT OF INHIBITION OF CYCLIC GMP PHOSPHODIESTERASE  
ON BLOOD-BRAIN BARRIER IN AN IN VIVO TEST SYSTEM

The in vivo tracer experiment of Example 11 was repeated, except that the test compound was the cyclic  
 15 GMP phosphodiesterase inhibitor, dipyridamole.

Table 10DPM/gm tissue

	<u>Condition</u>	<u>Time</u> min.	<u><sup>3</sup>H-sucrose</u>	<u><sup>125</sup>I-BSA</u>
20	Control	15	8,757 ± 2034	616 ± 46
	Dipyridamole	15	18,233 ± 4770	1110 ± 187
	Control	60	12,164 ± 1774	579 ± 47
	Dipyridamole	60	17,932 ± 2642	943 ± 157

25

The results show that inhibition of cyclic GMP phosphodiesterase, which results in an elevation of intracellular levels of cyclic GMP, increased the

- 0 transport across the blood-brain barrier of both small  
(sucrose) and large (bovine serum albumin) molecules.

#### EXAMPLE 18

##### EFFECTS OF DIFFERENT CLASSES OF PHOSPHODIESTERASE INHIBITORS ON BRAIN

##### 5 MICROVASCULAR ENDOTHELIAL CELL TIGHT JUNCTIONS IN VITRO

Bovine brain microvascular endothelial cells were isolated and grown on permeable filters in the BBB in vitro model of the invention. Cells were maintained in astrocyte-derived conditioned medium, but were not  
10 treated with agents that elevate cyclic AMP levels in such cells.

At the beginning of the experiment (i.e., in a low resistance state), cells were either left untreated (blk in Figure 7) or treated with 17.5  $\mu$ M Rolipram or  
15 RO-20-1724 (RO in Figure 7) (specific inhibitors of cyclic AMP phosphodiesterase). Resistance was then measured at various times. In both experiments (Figure 7), by 30 minutes resistance was already substantially higher in cells treated with these compounds. In  
20 contrast, specific inhibitors of cyclic GMP phosphodiesterase, zaprinast, dipyridamole and milrinone, were ineffective under analogous conditions. This suggests that the phosphodiesterase predominantly responsible for degrading cyclic AMP in brain  
25 endothelial cells is a Type III cyclic GMP-noninhibitable phosphodiesterase. This also suggests that inhibitors of this class of enzyme may be effective in treating vasogenic brain edema.

0

## EXAMPLE 19

RESISTANCE OF HUMAN BRAIN MICROVASCULAR ENDOTHELIAL  
CELLS IN AN IN VITRO BLOOD-BRAIN BARRIER MODEL

Brain biopsy specimens from human epileptic patients were transported from the operating room to the laboratory in a MEM-antibiotic medium. After dissecting away the meninges, gray matter was rinsed, then homogenized in L-15 medium as described above. The homogenate was passed through a 50  $\mu$ m nylon filter; the residue was passed through a 50  $\mu$ m filter twice more. The final residue was spun down, then suspended in 5 ml of a solution containing collagenase, trypsin and DNase, as described above for bovine brain endothelial cells. Isolated capillary fragments and cells were centrifuged, resuspended and plated on collagen-fibronectin-coated flasks in a growth medium containing rat astrocyte-derived conditioned medium. The cells were then maintained as described above for bovine brain cells.

After the cells had been transferred to permeable supports and grown to confluency, some were treated with chlorophenyl-thio-cyclic AMP + RO-20-1724 as detailed above.

Prior to elevation of cyclic AMP levels, resistance of the cells (average of six replicates) was 62.5 ohm-cm<sup>2</sup>. Subsequent to treatment, resistance of the cells (average of six replicates) averaged 357.8 ohm-cm<sup>2</sup>.

This experiment demonstrated that human brain microvascular endothelial cells responded as did the counterpart cells from bovine brain in terms of tight junction formation in response to cyclic AMP.

0

## EXAMPLE 20

EFFECT OF REMOVAL OF CYCLIC  
AMP-ELEVATED AGENTS ON THE SUBSEQUENT RESISTANCE  
OF BRAIN MICROVASCULAR ENDOTHELIAL CELLS

Bovine brain endothelial cells were grown to  
 5 confluency on filters in the blood-brain barrier of the  
 invention using the standard protocol described in  
 Examples 1, 3 and 5. Thereafter, cells were treated  
 for 48 hours with chlorophenyl-thio-cyclic AMP +  
 RO-20-1724. The medium containing these cyclic AMP  
 10 elevating agents was removed, and the cells were washed  
 with fresh growth medium. To some cells was added  
 growth medium alone, and to other cells was added  
 growth medium containing the cyclic AMP analogue and  
 RO-20-1724. The resistance of the cell monolayers was  
 15 measured periodically, and the results are shown in  
 Table 11.

Table 11

		<u>Resistance</u>	
20	<u>Time of</u> <u>second incubation</u> min.	<u>Medium alone</u>	<u>Medium +</u>
		ohm-cm <sup>2</sup>	"cyclic AMP" (average)
	Initial	322	322
	15	74	212
	30	69	284
25	60	59	293
	Overnight	39	332

Resistance dropped quite rapidly when  
 intracellular levels of cyclic AMP were reduced (Medium  
 30 alone in Table 11), due, probably, to the presence of a  
 rapidly acting phosphoprotein phosphatase that



0 dephosphorylates cyclic AMP-activated proteins.  
Resistance remained high when washed cells continued to  
be exposed to sources of intracellular cyclic AMP  
(Medium + "cyclic AMP").

## EXAMPLE 21

5 MODULATION OF LEUKOCYTE ADHESION TO  
INFLAMED BRAIN ENDOTHELIAL CELLS

In this preferred embodiment, antibodies against  
VLA-4 were shown to substantially prevent leukocyte  
adhesion to brain endothelial, using both a novel  
10 system for inducing MS-type inflammation in vivo, and  
the blood brain barrier model.

Unless otherwise stated, all technical and  
scientific terms used herein have the same meaning as  
commonly understood by one of ordinary skill in the art  
15 to which this invention belongs. Although any methods  
and materials similar or equivalent to those described  
herein can be used in the practice or testing of the  
present invention, the preferred methods and materials  
are now described. As set forth above, all  
20 publications to which reference is made are  
incorporated herein by reference.

Materials and Methods

Lymphocytes: Mouse or rat lymphocytes were  
isolated immediately before a binding assay from  
25 mesenteric, cervical and brachial lymph nodes by  
standard methods and crushed between the frosted ends  
of two glass slides. Human lymphocytes were isolated  
from heparinized or EDTA-treated whole blood using  
Mono/Poly separation medium (Flow Labs, Mclean, VA),  
30 and used immediately.

Lymphoid Cell Lines: All cell lines were obtained  
from the cell culture facility at the University of

0 California, San Francisco and were maintained in RPMI-  
1640 with 10% FBS (37°C, 10% CO<sub>2</sub>). RPMI-1460 was  
purchased from the University of California, San  
Francisco. These University of California cell lines  
are all made available to the public. Specifically, the  
5 cell lines obtained from the University of California,  
San Francisco are the Jurkat T-cell line, U937, THP-1,  
FRO, HL60, and HUT78. These cell lines may also be  
available from other sources.

Monoclonal Antibodies: AIIB2, against human  $\beta$ 1  
10 integrin (the "anti- $\beta$ -1"), is available from Dr.  
Caroline Damsky, Department of Oral Biology, University  
of California, San Francisco. HP2/1, against the VCAM-  
1-binding domain of VLA-4 (the "anti- $\alpha$ -4") was pur-  
chased from AMAC, Inc. (Westbrook ME, Product # 0764).  
15 The AP2/1 also cross-reacts with murine lymphocytes.  
P4H9, against human  $\beta$ 2 integrin (the "anti- $\beta$ -2") was  
purchased from Telios, Inc. (San Diego, CA Product  
#A052). This anti- $\beta$ -2, against the  $\beta$ -2 integrin is not  
known to react with any subunit of VLA-4.

20 When used to treat lymphocytes, the anti- $\beta$ -1  
hybridoma supernatant was used at a 1:2 dilution. The  
anti- $\alpha$ -4 antibody was purified by the manufacturer, and  
used at a 5  $\mu$ g/ml concentration. The anti- $\beta$ -2 was  
purified by the manufacturer, and used at 5  $\mu$ g/ml. For  
25 treatment of lymphocytes, the lymphocytes were mixed  
with the above concentrations of antibody, and allowed  
to incubate on ice for a about 30 minutes prior to use.  
The cells were washed to remove unbound antibody, and  
resuspended in RPMI to a typical concentration of 10<sup>7</sup>  
30 cells/ml.

Other tissue sections: In the in vitro frozen  
brain section assay, the preparation of brain sections  
is described below. Lymph node and intestinal tissues

0 were removed from rats, and sectioned as described for  
brain tissues, below.

A. In Vitro Frozen Brain Section Assay

In order to establish inflammatory brain lesions  
that involve a large degree of immune cell  
5 infiltration, rats were injected in the brain with  
human kidney cell line 293 (American Type Culture  
Collection, "ATCC," 1573). This method was found to  
stimulate the entry of all leukocyte classes into the  
brain in a predictable time course. The trauma of the  
10 injection induces the entry of neutrophils and  
monocytes within minutes, which continues for about 24  
to about 48 hours. The presence of the human cells  
serves as a persistent irritant to the immune system,  
stimulating further leukocyte infiltration, including  
15 that of lymphocytes. Typically, by about day 6,  
lymphocytes and monocytes are the major infiltrating  
leukocyte classes, entering in such large numbers so as  
to produce cellular cuffs around small blood vessels in  
the brain near the injection site. The speed and  
20 predictability of this procedure has made it ideal for  
obtaining brain tissue that can be used in the in vitro  
assay described below. In this assay, the brains are  
quickly frozen and sectioned. Leukocytes (immortalized  
and grown as cell lines, or freshly isolated from  
25 rodents or humans as described above) are then exposed  
to the sections, and, if they express the appropriate  
receptors, adhere selectively to the exposed profiles  
of activated endothelium near the inflammatory lesion.  
The leukocytes do not bind to nonactivated endothelium  
30 in the brain sections away from the inflammatory lesion  
or within sections of the non-stimulated control brain.

Rats (male Sprague-Dawley, 275-300g), were  
anesthetized with Nembutal (60 mg/kg-i.p.) and mounted

0 in a stereotaxic device. The head was shaved and an  
incision was made to reveal the dorsal skull. Holes  
were drilled through the skull on the left and right  
side overlying the parietal cortex.  $10^7$  human kidney  
derived cells (ATCC 1573 cell line), suspended in PBS,  
5 were delivered to the parietal cortices in a volume of  
 $10\mu\text{l}$ . It is believed that other allogeneic cells or  
cell lines would also induce the present MS-type  
symptoms via these methods. For example, we have used  
primary bovine microvascular endothelial cells to  
10 induce a similar inflammatory reaction.

The incision was sutured and the animal allowed to  
recover for 1-10 days. On the appropriate day, brains  
were removed from animals that had been anesthetized  
with halothane and killed by cardiac puncture. The  
15 cerebellum was removed and the brains were then placed  
rostral side down on a mound of gum tragacanth (mixed  
with water to the consistency of thick paste) and  
frozen by immersion for 60 seconds in 2-methyl butane  
chilled on dry ice. The brains were then stored in  
20 sealed tubes at  $-80^\circ\text{C}$ .

Immediately before the assay, 10 micron thick  
brain sections were cut on a cryostat and transferred  
to the center of a 14 mm well, pre-formed within a thin  
epoxy coating (Catalog #100314, Carlson Scientific,  
25 Inc., Peotone, IL) and allowed to air-dry at room  
temperature. The transfer was accomplished by touching  
the slide (at room temperature) to the section, which  
was still on the cold knife blade. Sections of control  
tissues (peripheral lymph nodes and Peyer's patches,  
30 isolated from noninjected rats, frozen and stored as  
described above) were usually placed adjacent to the  
brain sections in the same wells. The slides were  
positioned on a metal tray resting on ice and the wells

0 were filled with 100  $\mu$ l of the appropriate cell  
suspension. The metal tray and supporting ice were  
then gyrated at about 50- about 80 rpm for 30 minutes  
on an orbital shaker (Lab Line Instruments, Inc., Model  
3520, with 1 inch diameter rotation). The cell  
5 suspension was then decanted and the slides were  
carefully placed vertically in PBS with 2.5%  
glutaraldehyde on ice for 20 minutes. The slides were  
then dipped 5 times in PBS, placed in 0.5% toluidine  
blue (20% EtOH) for 1 minutes, destained with 2 brief  
10 dips in 100% ethanol, covered with Immu-mount<sup>TM</sup>  
mounting medium (Shandon, Sweickley, PA), and cover  
slipped.

Herein, cell suspensions used were freshly  
isolated rat, mouse or human lymphocytes, the U937T  
15 human myelomonocytic cell line ,and the Jurket human T  
cell line. Cell lines THP-1, FRO, HL60 and HUT78 were  
found not to bind with stimulated brain sections, and  
were not further analyzed in the brain section assay.

The degree of lymphocyte binding was quantified by  
20 one of two methods. The first relied upon an internal  
reference population of cells, similar to that  
described by Butcher, et al., J.Immunol 123: 1996-2003  
(1979). The second method was based on the absolute  
number of lymphocytes bound to blood vessels in a given  
25 tissue section. For the internal reference method,  
populations of lymphoid cell lines were mixed with  
freshly isolated lymphocytes of a different species  
(e.g. human cell lines with mouse lymphocytes) so that  
both were at a final concentration of  $3-5 \times 10^7$ /ml.  
30 Aliquots of the mixed population were then treated with  
species-specific antibodies for 30 minutes on ice. In  
general, the cells were washed out of the antibody  
prior to the binding assay. Binding was quantified by

0 determining the ratio of two different populations of  
 leukocytes bound to blood vessels. It was always  
 arranged such that the leukocyte populations could  
 readily be distinguished by size--lymphoid cell lines  
 are large cells, generally greater than 20  $\mu\text{m}$  in  
 5 diameter, while lymphocytes are small cells, less than  
 10  $\mu\text{m}$  in diameter. Thus in an experiment with human T  
 cell line mixed with rat lymphocytes, the degree of  
 inhibition produced by an anti-human monoclonal  
 antibody (compared to control antibodies or to no  
 10 treatment) was quantified by determining the ratio of  
 large to small cells bound. The results obtained are  
 presented in Table 12, below. As can be seen, use of  
 an anti-VLA-4 reagent significantly inhibited binding  
 of immune cells to brain cells displaying MS-type  
 15 inflammation. These results are also shown in Figure  
 10, which clearly displays the inhibitory effect of the  
 anti-VLA-4 reagents.

Table 12

Quantification of rat lymphocyte binding to different  
 20 tissue sections by comparing the number of lymphocytes  
 bound to all vessels under treated and untreated  
 conditions. Four replicates were used for each  
 treatment, and raw data are presented in parenthesis  
 below the mean.

25	<u>Treatment</u>	<u>Brain</u>	<u>Intestinal</u>	<u>Lymph Node</u>
	No treatment	24(100%) (24/24/32/18)	43(100%) (35/45/42/50)	21(100%) (25/27/18/15)
	anti-VLA- $\alpha$	2(8%) (2/3/2/2)	6(15%) (3/3/5/10)	20(95%) (14/30/22/12)

0       The second method of quantification compared a  
single population of leukocytes treated in different  
ways in adjacent assay wells. The degree of inhibition  
was determined by comparing the number of leukocytes  
bound to all vessels in a given tissue section under  
5       treated and untreated conditions. Tissue sections were  
prepared as described above. The degree of binding was  
quantified as the actual number of cells bound to the  
blood vessels within the sections. These data are  
presented in Table 13, below.

10       In addition, lymph node tissue was also tested,  
and these data are displayed in Figure 11. All  
reagents were prepared as described above. Here, anti-  
 $\beta$ -1 antibody and anti- $\alpha$ -4 antibody were both shown to  
inhibit Jurkat T-cell lymphocyte binding to brain  
15       sections, but not to lymph node sections.

These data confirm that anti-VLA-4 reagents showed  
substantial inhibition of leukocyte binding to brain  
tissue displaying the features of MS-type inflammation.

Table 13

20 <u>Treatment Used</u>	<u>Ratio large/small</u>	<u>% Control Jurkat Binding</u>
No treatment	$2.08 \pm 0.17$	$100 \pm 8$
anti- $\beta$ -1	$0.02 \pm 0.01$	$1 \pm 0.5$
anti- $\alpha$ -4	$0.23 \pm 0.15$	$11 \pm 7$

25       B.   Leukocyte Binding to Cultures of Blood Brain  
Barrier Endothelial Cells

Bovine or human brain endothelial cells were  
maintained in accordance with the blood brain barrier  
model as described in the present specification. In

0 experiments where the endothelium was activated, 5  $\mu$ l  
of stimulating agent was added directly to the lower  
chamber medium (800  $\mu$ l) of the culture system. Here,  
TNF $\alpha$  (Amgen Biologicals, Thousand Oaks, CA) was added  
to the lower chamber for a final concentration of 400  
5  $\mu$ /ml. In activation, PMA-S (in DMSO) was found not to  
be effective in stimulating lymphocyte binding, but  
other activating agents are known, and will be apparent  
to those skilled in the art.

In this manner, the endothelial cells were exposed  
10 to the agent on their ablumenal surface, as would be  
the typical situation during an inflammatory reaction  
in the brain. Immediately before the assay, the  
electrical resistance of the cultures was measured and  
the filters (supporting the endothelial cells) were  
15 washed at room temperature by dipping in three separate  
vats of D-MEM with 1% FBS and 20 mM Hepes (200 mls.  
each). The filters were then placed in fresh wells  
containing the same medium and the assay was performed  
at room temperature.

20 Typically, 10  $\mu$ l of leukocytes (at a preferred  
concentration of  $10^7$ /ml) in the presence or absence of  
test reagents, were added to the upper chamber of the  
culture system, such that the leukocytes would  
encounter the luminal or blood side of the endothelium,  
25 as they would in the brain vasculature. Here,  
leukocytes were rat, mouse or human lymphocytes, the  
U93T human myelomonocytic cell line, and the Jurket  
human T cell line as described above. The lymphocytes  
were pretreated with anti- $\beta$ -1 or anti- $\beta$ -2 antibody as  
30 described above.

Cell lines THP-1 and FRO were also found to bind  
to brain endothelial stimulated with TNF $\alpha$ , but HL60 and



0 HUT78 did not so bind. U937 binding was found not to be inhibited by exposure to anti- $\beta$ -1.

The culture plates were placed on a gyratory shaker at about 100 rpm for 30 seconds, then allowed to sit undisturbed at room temperature for about 30  
5 minutes. The assay was terminated by gently washing the filters in PBS with 1% glutaraldehyde (dipping and pouring three times at different angles). The glutaraldehyde causes the cells to fluoresce under the proper optical conditions, as described below. The  
10 filters were then allowed to fix in the glutaraldehyde solution undisturbed for 60 minutes.

The degree of leukocyte binding to the filters was examined in one of two ways. In the first, the bound cells were visualized directly. The filter was cut  
15 free of the culture well apparatus and mounting on glass slides with Immu-mount<sup>TM</sup>. The filters were examined with an immunofluorescence microscope set for rhodamine or fluorescein optics, and observing the cells by glutaraldehyde-induced autofluorescence.

20 The results of the immunofluorescence assay can be visualized in Figure 8. As is easily visualized, the density of Jurkat T-cell lymphocytes pretreated with anti- $\beta$ -1 antibody is far lower (Panel A) than the binding density for untreated leukocytes (Panel B).  
25 This graphically depicts the anatomical and physiological reaction when a reagent is used to block the VCAM-1/VLA-4 interaction between brain endothelial cells and leukocytes.

In the second method, the leukocytes were pre-  
30 labeled with a radioactive tracer and the degree of binding was quantified by measuring the amount of radioactivity associated with the entire endothelial surface on the culture filter. Prelabeling of lymphoid

0 cell lines was accomplished by the addition of 1 uCi/ml  
125IUDR (Amersham #XX) obtained from the Amersham  
Corporation, Arlington Heights, Illinois, to the  
standard culture medium approximately 12-20 hrs. before  
the assay. The cells were washed free of  
5 unincorporated label by three separate washes in 15 mls  
of fresh bench medium (RPMI-1640 with 5% FBS and 25 mM  
Hepes). Concentration was then adjusted to  $10^7$  cells  
/ml in the presence or absence of test reagents. Again,  
all of the above lymphocytes were used (rat, mouse or  
10 human lymphocytes, the U937T human myelomonocytic cell  
line, and the Jurket human T cell line) as described  
above. The lymphocytes were pretreated with anti- $\beta$ -1  
or anti- $\beta$ -2 antibody as described above. Also, the  
lymphocytes were pretreated with anti-VLA- $\alpha$ -4 as  
15 described above.

The assay was carried out as above, except that at  
the end the isolated filters are placed in tubes and  
counted in a gamma counter (Beckman Corporation, Model  
5500B) for 1 minute. The results are presented in  
20 Table 14 below. As can be seen, the samples that  
contained anti-VLA-4 reagents show far lower  
radioactivity levels the controls. These data confirm  
the results from the above fluorescence data, namely,  
that the binding of anti-VLA-4 to the VCAM-1 receptor  
25 is substantially inhibited by reagents which would  
prevent binding at that locus.

These data are also presented at Figure 11 which  
shows the relative degree of Jurkat T-cell lymphocyte  
binding to brain endothelial cells in the BBB system.  
30 As can easily be seen, the anti- $\beta$ -1 antibody  
effectively inhibited the binding of leukocytes to TNF-  
 $\alpha$  activated brain endothelial cells. Anti- $\beta$ -2, as a  
control, on the other hand, approaches the untreated

- 0 control. Plainly, the  $\beta$ -1 subunit provides an effective target for preventing VLA-4/VCAM-1 interaction in the brain.

Table 14

- This table shows the quantification of  
5 lymphocyte binding via radioactive labelling of lymphocytes.

	<u>Lymphocyte Type</u>	<u>Treatment</u>	<u>Count per Minute</u>
	Jurkat T-cell		
10		0 (no TNF $\alpha$ )	112; 273
		anti- $\beta$ -1 (no TNF $\alpha$ )	259; 156
		anti- $\beta$ -2 (no TNF $\alpha$ )	198; 124
		0 (+ TNF $\alpha$ )	1430; 1150
		anti- $\beta$ -1 (+ TNF $\alpha$ )	361; 385
15		anti- $\beta$ -2 (+ TNF $\alpha$ )	1313; (n/a)

- The above discussion of this invention is directed primarily to preferred embodiments and practices thereof. It will be readily apparent to those skilled in the art that further changes and modifications in  
20 the actual implementation of the concepts described herein can easily be made without departing from the spirit and scope of the invention as defined by the following claims.

We claim:

1. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells and a means for augmenting cyclic AMP concentrations in said cells such that peripheral phalloidin staining is substantially present.

2. A model as recited in claim 1, wherein said endothelial cells are mixed endothelial cells.

3. A model as recited in claim 1, wherein said porous solid support is a porous filter or membrane of a material selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

4. A model as recited in claim 1, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between a second coat of said endothelial cells and said porous solid support.

5. A model as recited in claim 4, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

6. A model as recited in claim 1, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

7. An in vitro model of a blood-brain barrier as recited in claim 1, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

8. An in vitro model of a blood-brain barrier as recited in claim 1, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

9. A model as recited in claim 1, wherein said porous solid support comprises microcarrier beads.

10. A model as recited in claim 9, further comprising a first coat of an extracellular matrix material disposed on said beads, interposed between said beads and said endothelial cells.

11. A model as recited in claim 10, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>®</sup>.

12. A model as recited in claim 1, wherein said porous solid support comprises a tubular hollow fiber.

13. A model as recited in claim 12, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

14. A model as recited in claim 13, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte

extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

15. A model as recited in claim 1, wherein E-cadherin is substantially present between endothelial cells.

16. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells and a means for augmenting effective cyclic AMP in such cells, such that the transmonolayer resistance is at least about 200 ohm-cm<sup>2</sup>.

17. A model as recited in claim 16, wherein said endothelial cells are mixed or cloned endothelial cells.

18. A model as recited in claim 16, wherein said porous solid support is a porous filter or membrane selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

19. A model as recited in claim 16, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between said endothelial cells and said porous solid support.

20. A model as recited in claim 19, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

21. A model as recited in claim 16, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate

cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

22. An in vitro model of a blood-brain barrier as recited in claim 16, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

23. An in vitro model of a blood-brain barrier as recited in claim 16, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

24. A model as recited in claim 16, wherein said porous solid support comprises a tubular hollow fiber.

25. A model as recited in claim 24, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

26. A model as recited in claim 25, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

27. A model as recited in claim 16, wherein E-cadherin is substantially present between endothelial cells.

28. An in vitro model of a blood-brain barrier comprising a porous solid support upon which is

disposed an essentially confluent monolayer of brain microvascular endothelial cells, a means for augmenting effective cyclic AMP concentrations in said cells, and a means for growing the brain microvascular endothelial cells in a cell-derived conditioned medium or the equivalent, such that peripheral phalloidin staining is substantially present.

29. A model as recited in claim 28, wherein said endothelial cells are mixed or cloned endothelial cells.

30. A model as recited in claim 28, wherein said porous solid support is a porous filter or membrane selected from among the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

31. A model as recited in claim 28, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between said endothelial cells and said porous solid support.

32. A model as recited in claim 31, wherein said first coat extracellular matrix is selected from among the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

33. A model as recited in claim 28, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.



34. A model as recited in claim 28 wherein said cell-derived conditioned medium comprises endothelial cell-derived conditioned medium or astrocyte-derived conditioned medium, and said equivalent comprises a tissue extract.

35. An in vitro model of a blood-brain barrier as recited in claim 28, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

36. An in vitro model of a blood-brain barrier as recited in claim 28, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

37. A model as recited in claim 28, wherein said porous solid support comprises microcarrier beads.

38. A model as recited in claim 37, further comprising a first coat of an extracellular matrix material disposed on said beads, interposed between said beads and said endothelial cells.

39. A model as recited in claim 38, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>®</sup>.

40. A model as recited in claim 28, wherein said porous solid support comprises a tubular hollow fiber.

41. A model as recited in claim 40, further comprising a first coat of an extracellular matrix

material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

42. A model as recited in claim 41, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

43. A model as recited in claim 28, wherein E-cadherin is substantially present between endothelial cells.

44. An in vitro model of a blood brain barrier comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells, a means for augmenting effective cyclic AMP concentrations in said cells and a means for growing said endothelial cells in a cell-derived conditioned medium or the equivalent, such that peripheral phalloidin staining is substantially present, and having a trans-monolayer resistance of at least about 200 ohm-cm<sup>2</sup>.

45. A model as recited in claim 44, wherein said endothelial cells are mixed or cloned endothelial cells.

46. A model as recited in claim 44, wherein said porous solid support is a porous filter or membrane selected from among the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

47. A model as recited in claim 44, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between said endothelial cells and said porous solid support.

48. A model as recited in claim 47, wherein said first coat extracellular matrix material is selected from among the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

49. A model as recited in claim 44, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

50. A model as recited in claim 44, wherein said cell-derived conditioned medium comprises endothelial cell-derived or astrocyte-derived conditioned medium, and said equivalent comprises a cell extract.

51. An in vitro model of a blood-brain barrier as recited in claim 44, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

52. An in vitro model of a blood-brain barrier as recited in claim 44, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

53. A model as recited in claim 44, wherein said porous solid support comprises a tubular hollow fiber.

54. A model as recited in claim 53, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

55. A model as recited in claim 54, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

56. A model as recited in claim 44, wherein E-cadherin is substantially present between endothelial cells.

57. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells and a means for augmenting effective cyclic AMP concentrations in said cells such that peripheral phalloidin staining is substantially present, and having a transmonolayer electric resistance of at least about 200 ohm-cm<sup>2</sup>.

58. A model as recited in claim 57, wherein said endothelial cells are mixed endothelial cells.

59. A model as recited in claim 57, wherein said porous solid support is a porous filter or membrane selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

60. A model as recited in claim 57, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between a second coat of said endothelial cells and said porous solid support.

61. A model as recited in claim 60, wherein said first coat extracellular matrix material is

selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

62. A model as recited in claim 57, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

63. An in vitro model of a blood-brain barrier as recited in claim 57, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

64. An in vitro model of a blood-brain barrier as recited in claim 57, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

65. A model as recited in claim 57, wherein said porous solid support comprises a tubular hollow fiber.

66. A model as recited in claim 65, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

67. A model as recited in claim 66, wherein said first coat extracellular matrix material is

selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

68. A model as recited in claim 57, wherein E-cadherin is substantially present between endothelial cells.

69. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells, a means for augmenting effective cyclic AMP concentrations in said endothelial cells, and a means for growing said cells in a cell-derived conditioned medium or the equivalent, such that the transmonolayer electrical resistance is at least about 200 ohm-cm<sup>2</sup>.

70. A model as recited in claim 69, wherein said endothelial cells are mixed or cloned endothelial cells.

71. A model as recited in claim 69, wherein said porous solid support is a porous filter or membrane selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

72. A model as recited in claim 69, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between a second coat of said endothelial cells and said porous solid support.

73. A model as recited in claim 72, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

74. A model as recited in claim 69, wherein said means for augmenting effective cyclic AMP concentration in said endothelial cells is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

75. A model as recited in claim 69, wherein said cell-derived conditioned medium comprises endothelial cell-derived or astrocyte-derived conditioned medium, and said equivalent comprises a tissue extract.

76. An in vitro model of a blood-brain barrier as recited in claim 69, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

77. An in vitro model of a blood-brain barrier as recited in claim 69, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

78. A model as recited in claim 69, wherein said porous solid support comprises a tubular hollow fiber.

79. A model as recited in claim 78, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

80. A model as recited in claim 79, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel<sup>R</sup>.

81. A model as recited in claim 69, wherein E-cadherin is substantially present between endothelial cells.

82. A model as recited in claim 1, wherein said microvascular endothelial cells are brain capillary cells.

83. A model as recited in claim 16, wherein said microvascular endothelial cells are brain capillary cells.

84. A model as recited in claim 28, wherein said microvascular endothelial cells are brain capillary cells.

85. A model as recited in claim 44, wherein said microvascular endothelial cells are brain capillary cells.

86. A model as recited in claim 57, wherein said microvascular endothelial cells are brain capillary cells.

87. A model as recited in claim 69, wherein said microvascular endothelial cells are brain capillary cells.

88. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for said drug to cross the permeability barrier created by such junctions, comprising an agent capable of reducing the effective intracellular concentration of cyclic AMP, so that cell-cell adhesion is disrupted.



89. The composition of claim 88, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

90. The composition of claim 88, wherein said agent is selected from among the group consisting of agents that reduce adenylate cyclase activity, agents that inhibit the functioning of  $G_s$  agonists, agents that stimulate the degradation of cyclic AMP or cyclic AMP analogues or derivatives, and agents that inhibit the penetration of cells by cyclic AMP or analogues or derivatives of cyclic AMP.

91. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for a drug to cross the permeability barrier created by such junctions, comprising an agent capable of reducing the physiological effects of cyclic AMP, so that cell-cell adhesion is disrupted.

92. The composition of claim 91, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

93. The composition of claim 91, wherein said agent is an agent that inhibits cyclic AMP-activatable protein kinase or stimulates a phosphoprotein phosphatase.

94. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for a drug to cross the permeability barrier created by such functions, comprising an agent capable of elevating the effective intracellular concentration of cyclic GMP, so that cell-cell adhesion is disrupted.

95. The composition of claim 94, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

96. The composition of claim 94, wherein said agent is selected from among the group consisting of an agent that elevates guanylate cyclase activity, an agent that decreases the degradation of cyclic GMP or cyclic GMP analogues or derivatives, and an agent that stimulates the entry into cells of cyclic GMP or analogues or derivatives of cyclic GMP.

97. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for a drug to cross the permeability barrier created by such junctions, comprising an agent capable of increasing the physiological effects of cyclic GMP, so that cell-cell adhesion is disrupted.

98. The composition of claim 97, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

99. The composition of anyone of claims 88, 91, 94 and 97 in a pharmaceutically acceptable vehicle.

100. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the steps of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of reducing the effective intracellular concentration of cyclic AMP, so that cell-cell adhesion is disrupted and whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

101. The method of claim 100, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

102. The method of claim 100, wherein said agent is selected from among the group consisting of agents that reduce adenylate cyclase activity, agents that inhibit the functioning of  $G_s$  agonists, agents that stimulate the degradation of cyclic AMP or cyclic AMP analogues or derivatives, and agents that inhibit the penetration of cells by cyclic AMP or analogues or derivatives of cyclic AMP.

103. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the step of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of reducing the physiological effects of cyclic AMP, so that cell-cell adhesion is disrupted and whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

104. The method of claim 103, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

105. The method of claim 103, wherein said agent is an agent that inhibits cyclic AMP-activatable protein kinase or stimulates a phosphoprotein phosphatase.

106. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the steps of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of elevating the effective intracellular concentration of cyclic GMP, so that cell-cell adhesion is disrupted and

whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

107. The method of claim 106, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

108. The method of claim 106, wherein said agent is selected from among the group consisting of an agent that elevates guanylate cyclase activity, an agent that decreases the degradation of cyclic GMP or cyclic GMP analogues or derivatives, and an agent that stimulates the entry into cells of cyclic GMP or analogues or derivatives of cyclic GMP.

109. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the steps of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of increasing the physiological effects of cyclic GMP, so that cell-cell adhesion is disrupted and whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

110. The method of claim 109, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

111. A method of treating vasogenic brain edema in a subject, comprising the step of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of increasing the physiologically-effective intracellular concentration of cyclic AMP, so that means are provided for increasing tight junctions between brain microvascular endothelial cells and so that cell tight junction permeability is reduced.

112. The method of claim 111, wherein said agent is selected from among the group consisting of agents that increase cellular adenylate cyclase activity, agents that inhibit the degradation of cellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

113. A method of treating vasogenic brain edema in a subject, comprising the step of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of increasing the physiological effects of cyclic AMP, so that means are provided for increasing tight junctions between brain microvascular endothelial cells and so that cell tight junction permeability is reduced.

114. The method of claim 113, wherein said agent comprises a protein kinase stimulator or a phosphoprotein phosphatase inhibitor.

115. A composition for treating vasogenic brain edema in a subject, whereby means are provided for inhibiting tight junction permeability of brain microvascular endothelial cells, comprising an agent, in a pharmaceutically-acceptable vehicle, capable of increasing the physiologically-effective intracellular concentration of cyclic AMP.

116. The composition of claim 115, comprising agents selected from among the group consisting of agents that increase cellular adenylate cyclase activity, agents that inhibit the degradation of cellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

117. A composition for treating vasogenic brain edema in a subject, whereby means are provided for inhibiting tight junction permeability of brain microvascular endothelial cells, comprising an agent, in a pharmaceutically-acceptable vehicle, capable of increasing the physiological effects of cyclic AMP.

118. The composition of claim 117, wherein said agent comprises a protein kinase stimulator or a phosphoprotein phosphatase inhibitor.

119. A method for modulating leukocyte adhesion to brain endothelial cells by substantially preventing a leukocyte VLA-4 molecule from binding to a brain endothelial ~~VCAM-1 molecule~~. *ligand.*

120. The method of claim 119 wherein said method includes use of a reagent that blocks the binding of said VLA-4 molecule to said VCAM-1 molecule.

121. A method of claim 119 wherein said reagent is a receptor.

122. A method of claim 121 wherein said receptor is selected from the group consisting of an antibody, an antibody fragment thereof, and a peptide.

123. A method of claim 121 wherein said receptor is directed against a VLA-4 molecule.

124. A method of claim 123 wherein said receptor is directed against the  $\alpha$ -1 subunit of the VLA-4 cell adhesion molecule.

125. A method of claim 121 wherein said receptor is directed against both the  $\alpha$ -4 and  $\beta$ -1 subunits of the VLA-4 adhesion molecule.

126. A method of claim 121 wherein said receptor is directed against a VCAM-1 molecule.

127. A method of claim 121 wherein said receptor is a monoclonal antibody.

128. A method for substantially preventing or ameliorating brain inflammation including modulating leukocyte VLA-4 molecule adhesion to brain endothelial cell VCAM-1 molecules.

129. A method of claim 128 wherein said method includes use of a reagent which blocks the binding of said VLA-4 molecule to said VCAM-1 molecule.

130. A method of claim 129 wherein said reagent is a receptor.

131. A method of claim 130 wherein said receptor is selected from the group consisting of an antibody, an antibody fragment thereof, and a peptide.

132. A method of claim 130 wherein said receptor is directed against the VLA-4 molecule.

133. A method of claim 132 wherein said receptor is directed against the  $\alpha$ -1 subunit of the VLA-4 molecule.

134. A method of claim 130 wherein said receptor is directed against both the  $\alpha$ -4 and  $\beta$ -1 subunits of the VLA-4 molecule.

135. A method of claim 130 wherein said receptor is directed against a VCAM-1 molecule.

136. A method of claim 130 wherein said receptor is a monoclonal antibody.

137. In a method for treating chronic inflammatory brain disease, the improvement comprising administering a therapeutically effective dosage of a reagent that substantially prevents a leukocyte VLA-4 molecule from binding to a brain endothelial VCAM-1 molecule.

138. A method of claim 137 wherein said reagent is a receptor.

139. A method of claim 138 wherein said receptor is selected from the group consisting of an antibody, an antibody fragment thereof, and a peptide.

140. A method of claim 138 wherein said receptor is directed against the VLA-4 molecule.

141. A method of claim 140 wherein said receptor is directed against the  $\alpha$ -1 subunit of the VLA-4 molecule.

142. A method of claim 138 wherein said receptor is directed against both the  $\alpha$ -4 and  $\beta$ -1 subunits of the VLA-4 molecule.

143. A method of claim 138 wherein said receptor is directed against a VCAM-1 molecule.

144. A method of claim 138 wherein said receptor is a monoclonal antibody.

145. A composition for preventing or ameliorating brain inflammation including at least one reagent that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1 molecule.

146. A composition in therapeutically effective form for preventing or ameliorating brain inflammation including at least one receptor that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1 molecule.

147. A composition for treating brain inflammation including a therapeutically effective form of at least one receptor that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1 molecule.

148. A composition for treating inflammation including a therapeutically effective form of at least one receptor that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1



molecule and delivers to the brain endothelial cell an anti-inflammatory agent.

149. A method for inducing brain inflammation via the intra-cranial injection of tumor cells.

150. The method of claim 149 wherein said tumor cells are ATCC 1573 human kidney-derived.

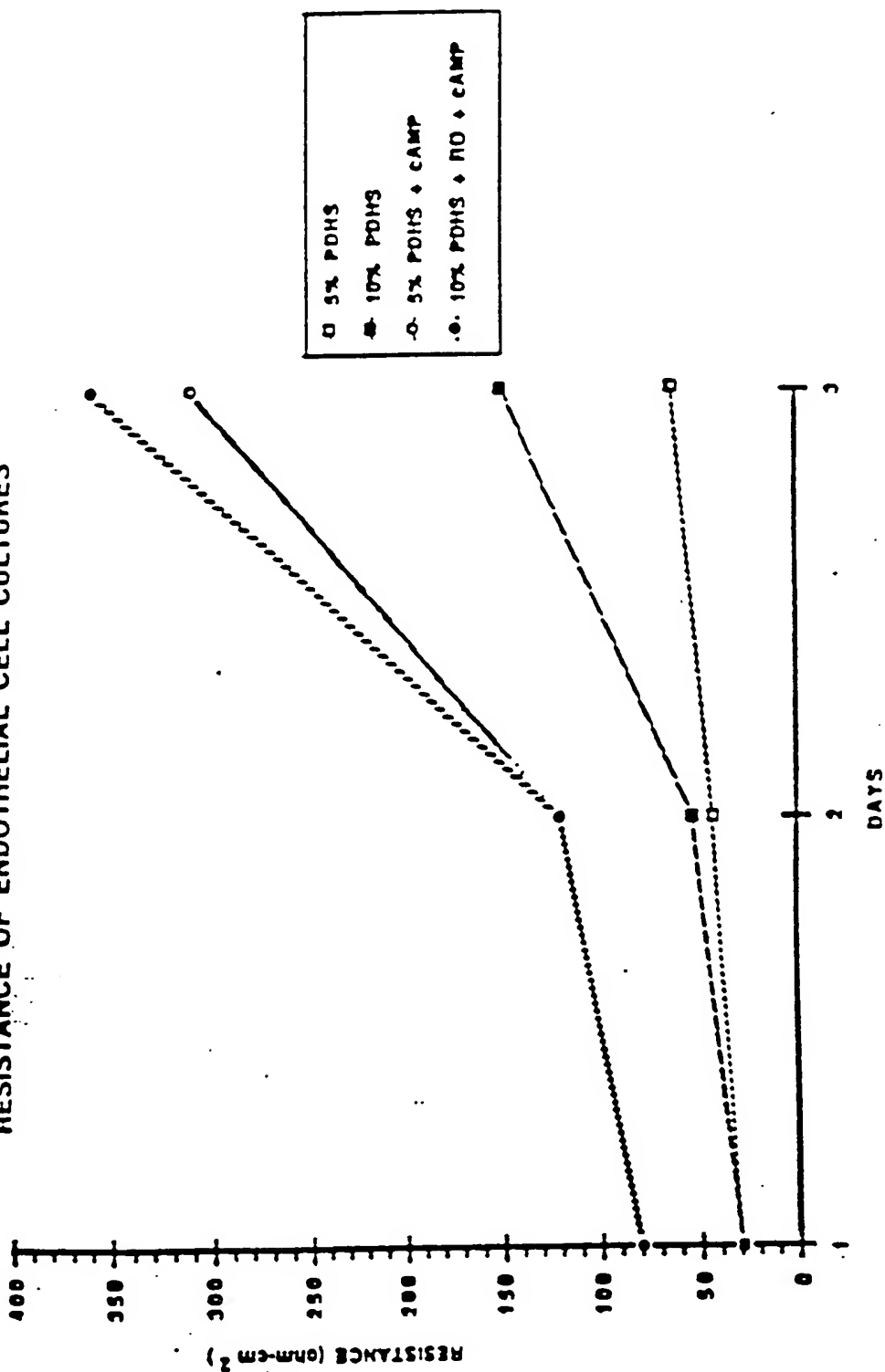
151. The method of claim 149 wherein a rat is injected intra-cranially.

152. An assay for ascertaining whether a compound is effective as an anti-inflammatory agent in the brain, wherein said assay includes inducing brain inflammation in a living organism via the intra-cranial injection of tumor cells, exposing brain tissue from said injected living organism to said putative brain anti-inflammatory agent.

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Fig. 1

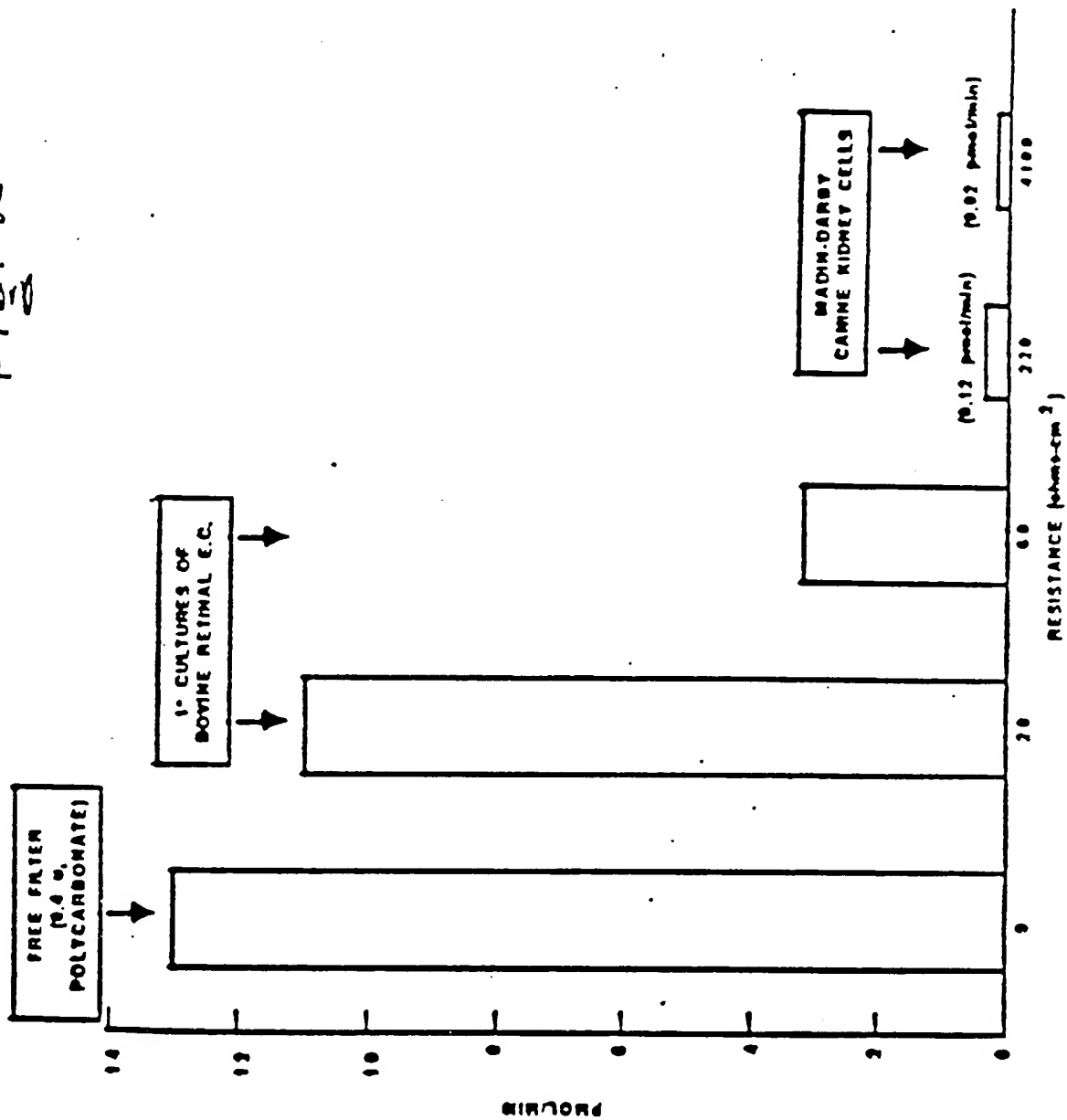
RESISTANCE OF ENDOTHELIAL CELL CULTURES



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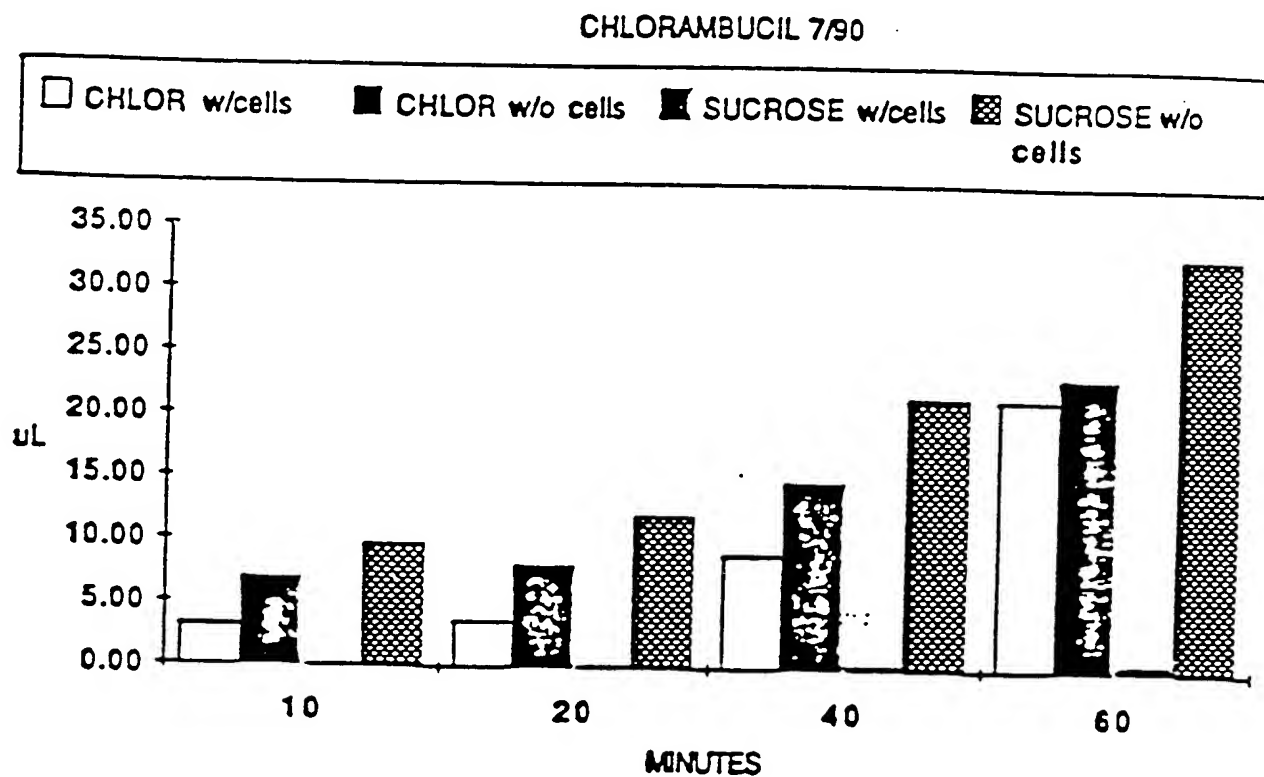
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Fig. 2



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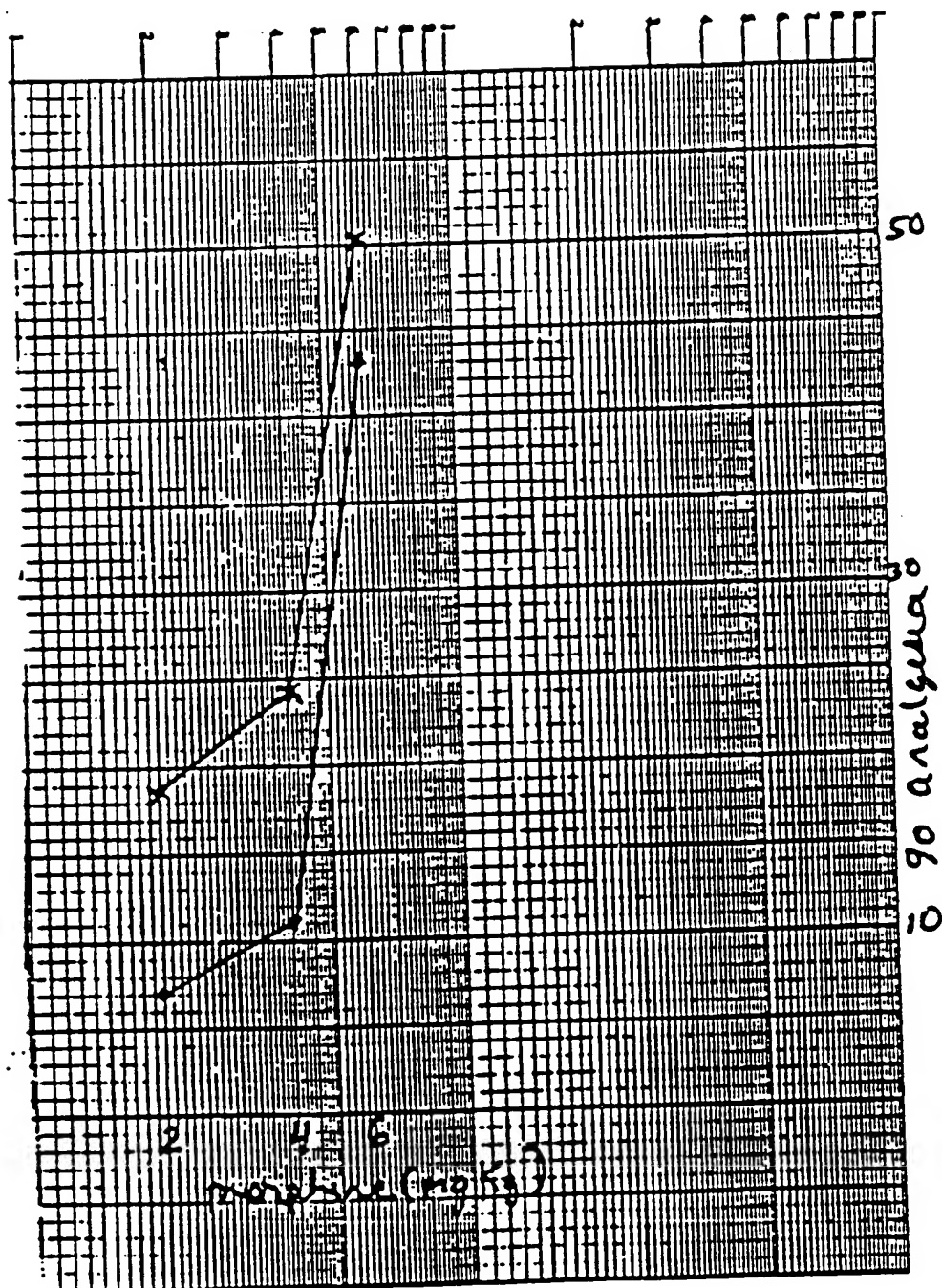
Fig. 3



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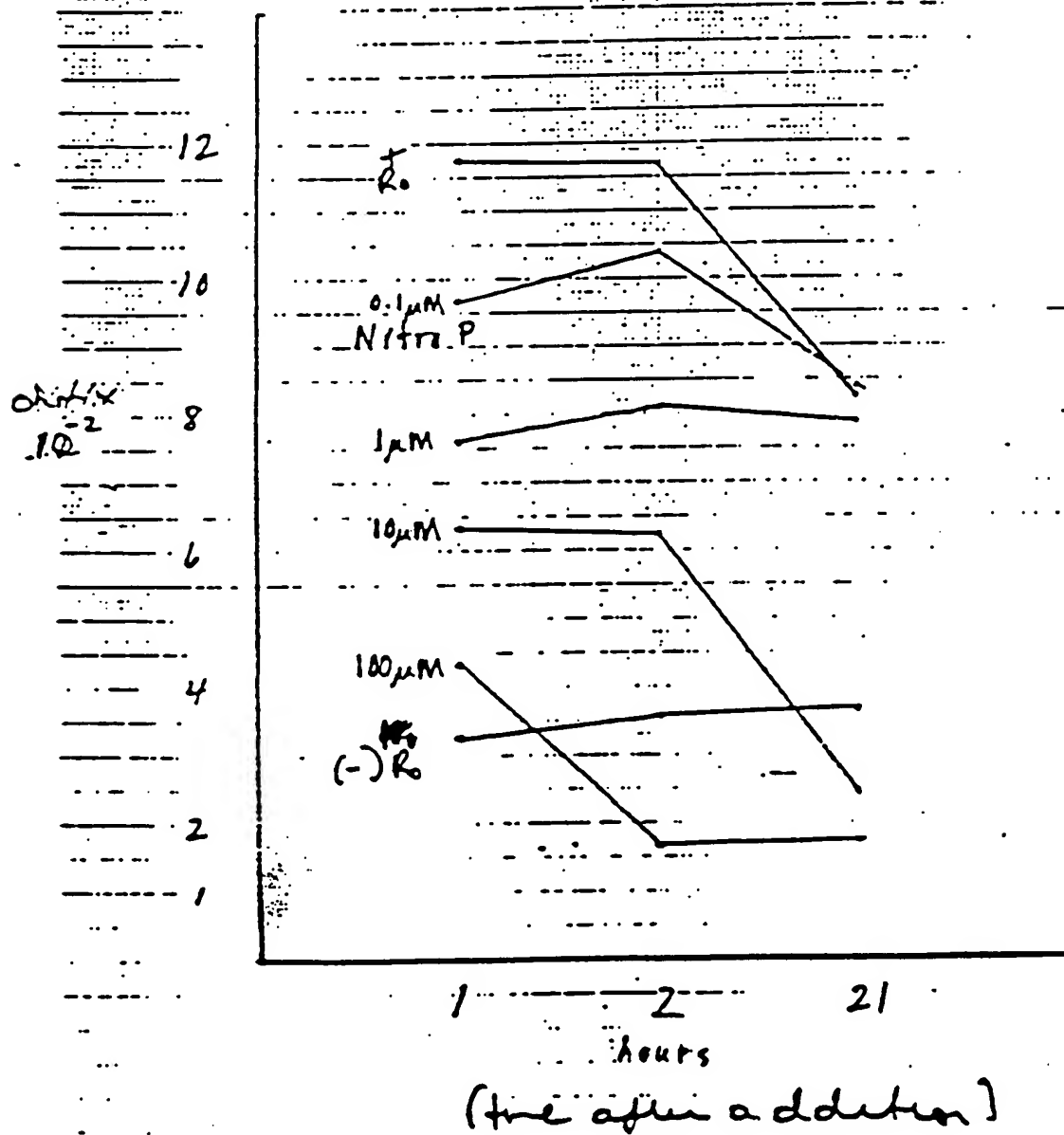
Fig. 4



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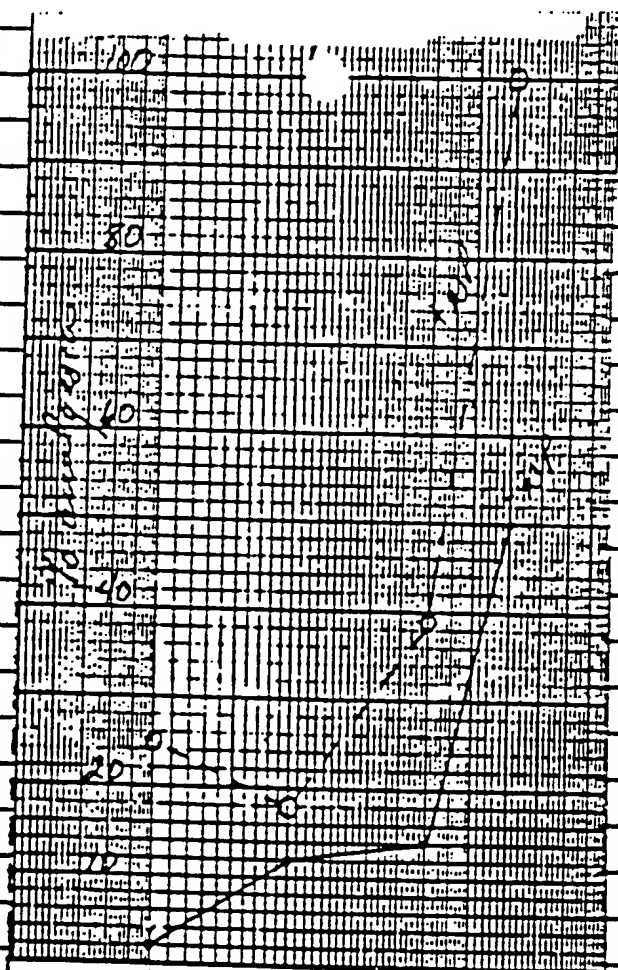
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Fig. 5



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Figure 6

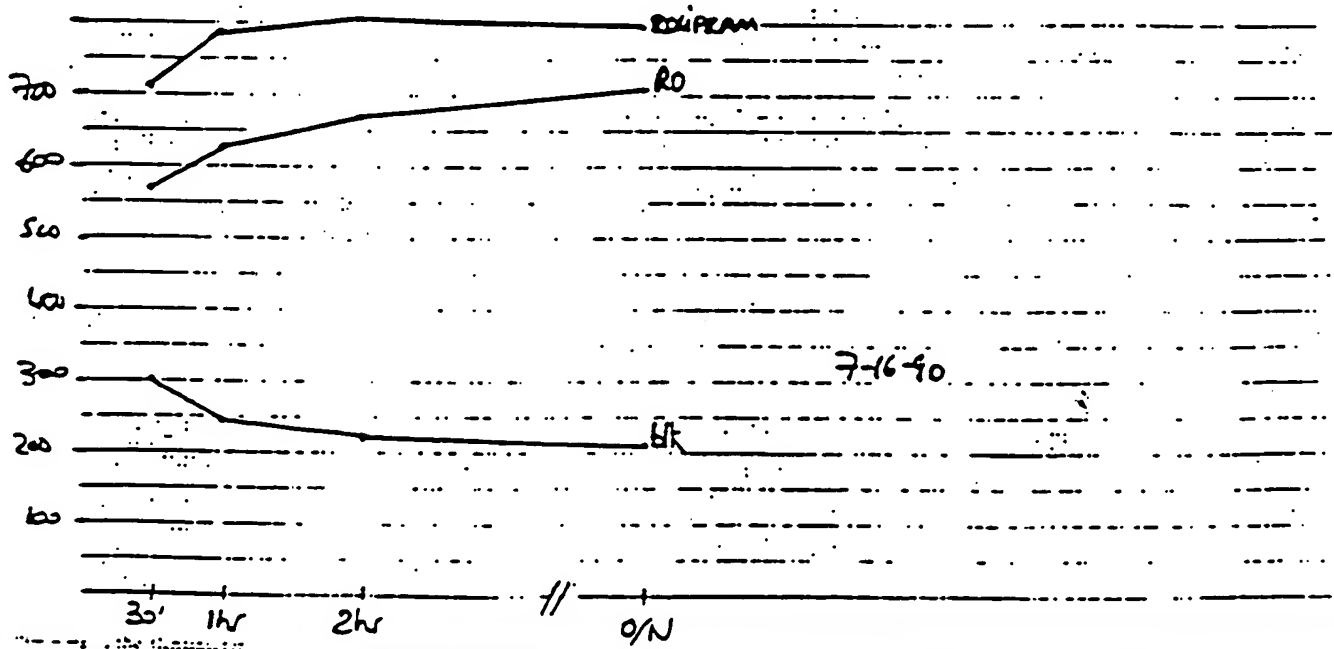
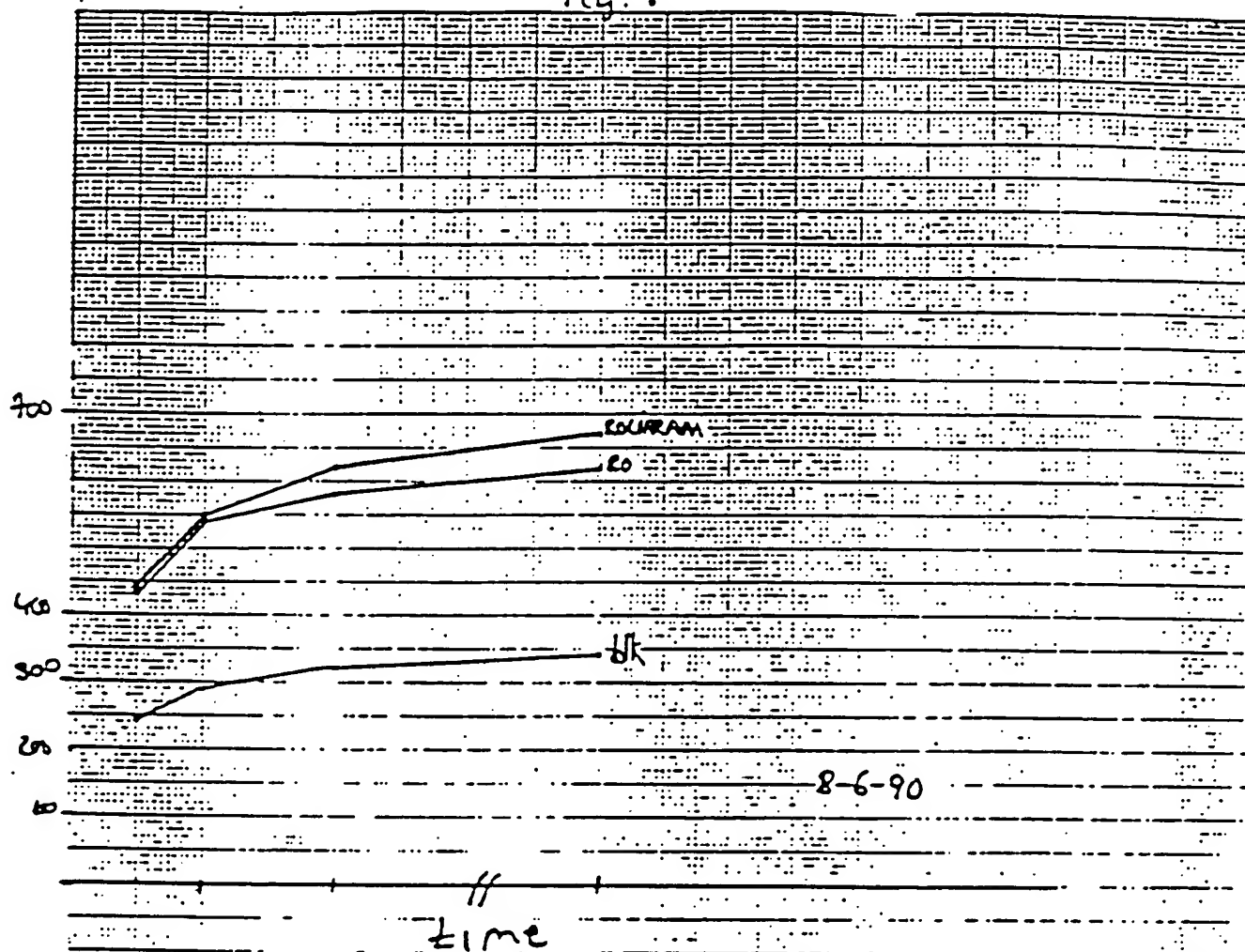
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Fig. 7<sup>7/11</sup>

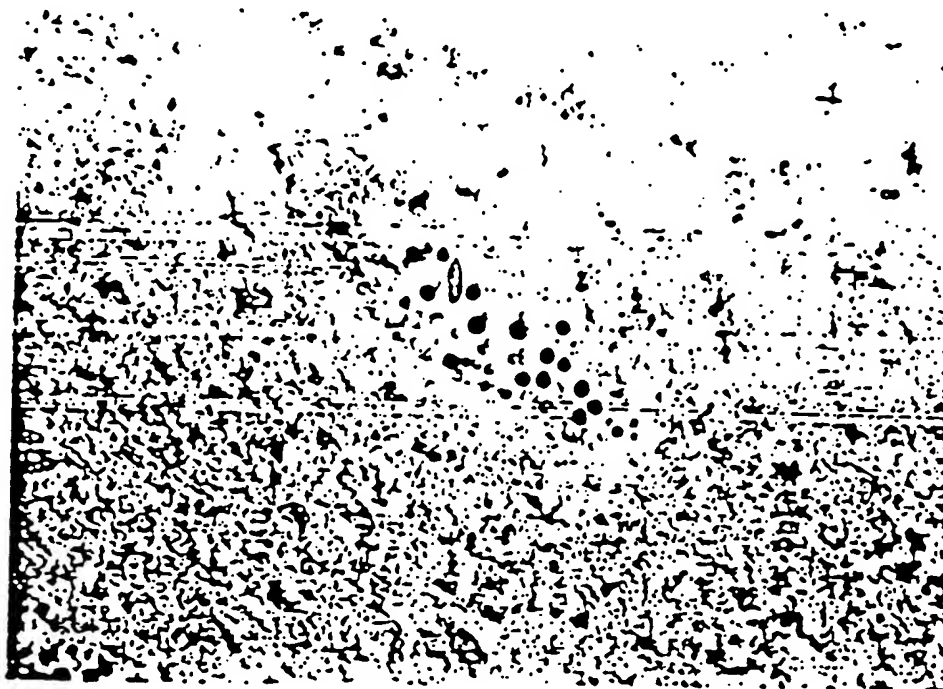


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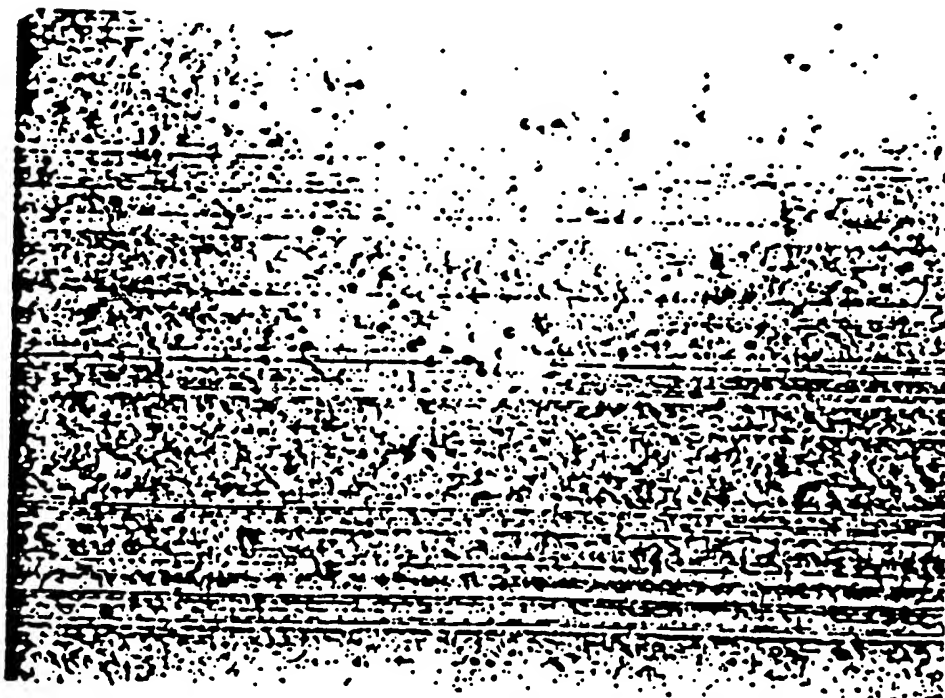


**Figure 8: Brain Section Assay**

**A. No Treatment** Binding of mixed human (large cell) and mouse lymphocyte (small cell) populations (see Methods).



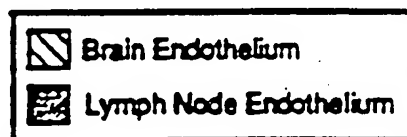
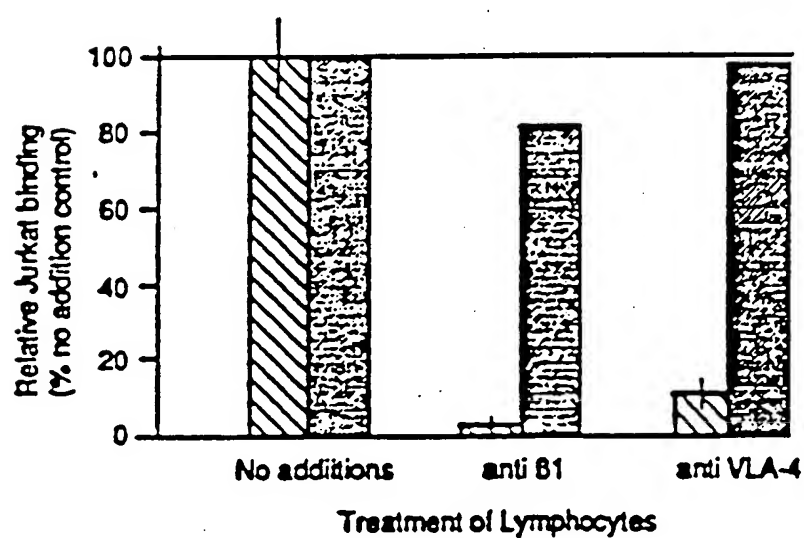
**B. Anti - B1 Integrin** Mixed lymphocyte population as in A, treated with anti-human B1. Binding of large, human cells is inhibited.



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Figure 10. Lymphocyte Binding to Blood Vessels In Sections of Lymph Node and Inflamed Brain



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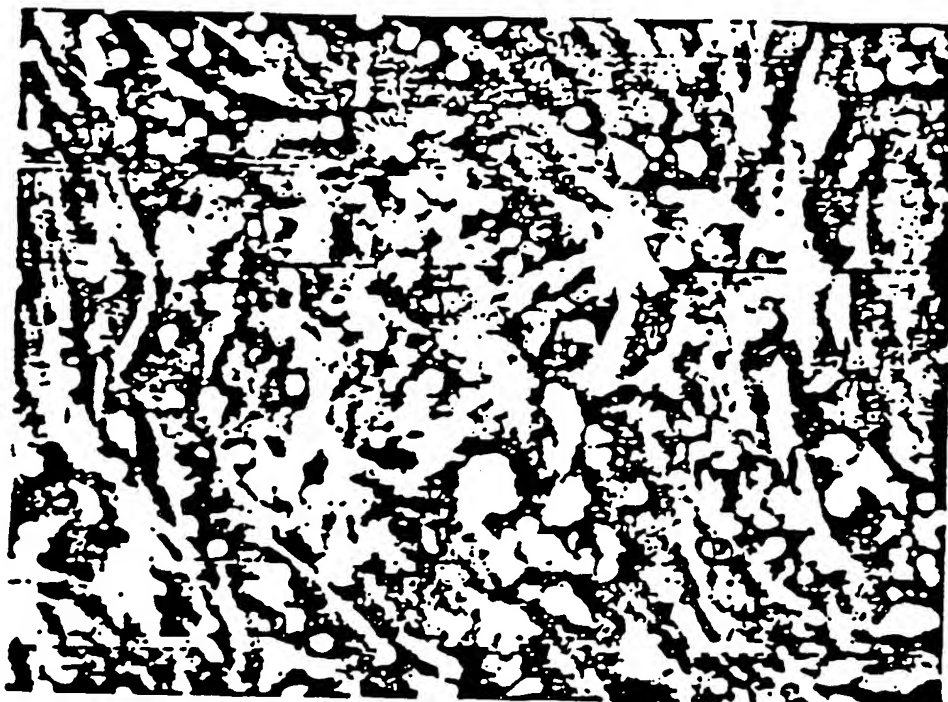
**Figure 9: Cultured BBB Endothelium Assay**

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**A. Low level binding of human lymphocytes to untreated endothelium**



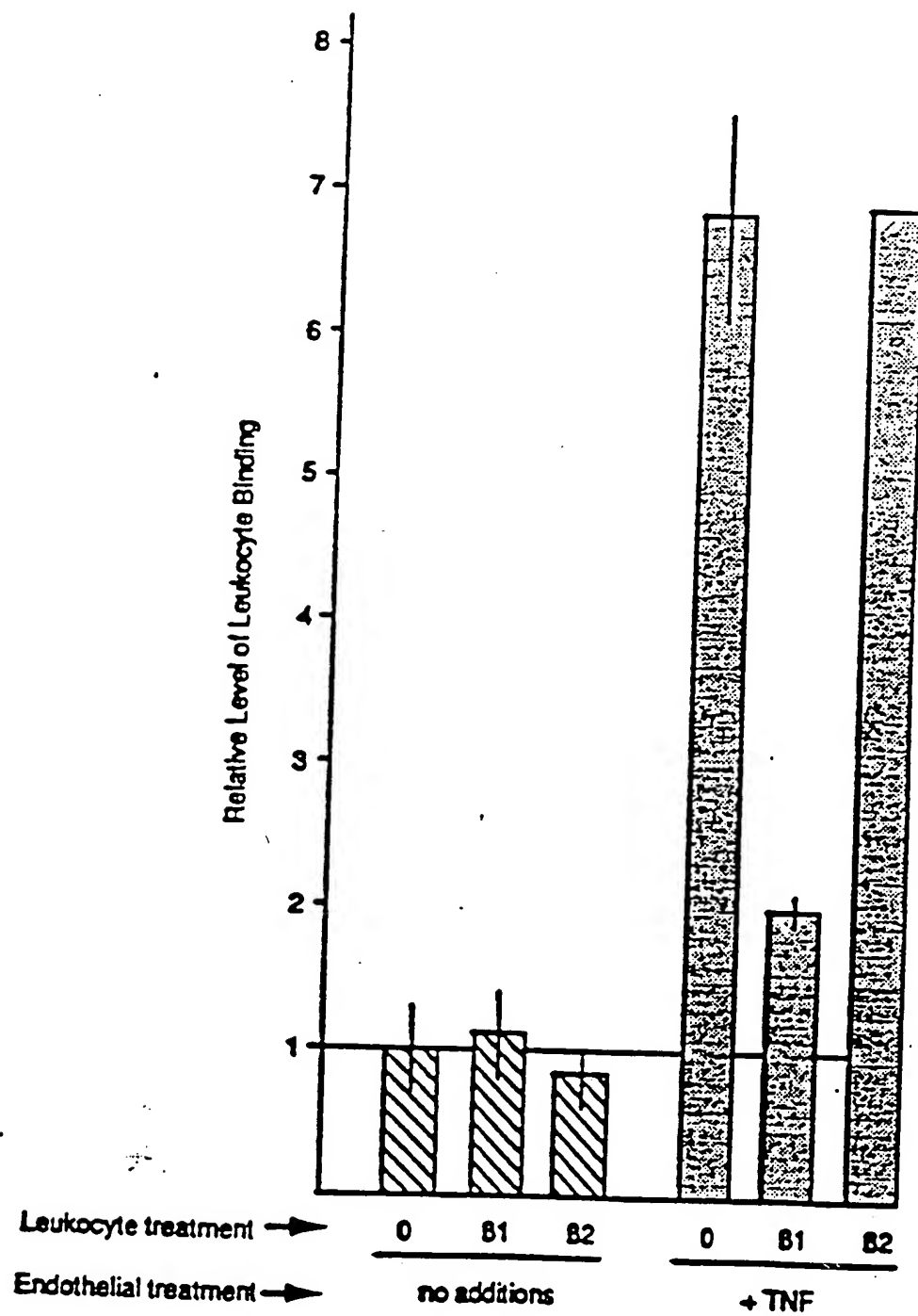
**B. Human lymphocytes bind approximately 7-fold better to endothelium stimulated with TNF**



**C. Treatment of human lymphocytes with anti- $\alpha 1$  Integrin inhibits their augment binding to TNF-stimulated endothelium**



Figure 1: Jurkat Binding to Brain EC



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